Development of a novel expression system for the production of recombinant proteins in Chinese hamster ovary cells based on the selection with a metabolic enzyme

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Content

Abbreviations

A	Adenine
ACSD	Affinity capture surface display
Ad-tpl	Adenovirus tripartite leader
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADP	Adenosine diphosphate
APC	Allophycocyanin
Asn	Asparagine
Asn	Asparagine
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BGH	Bovine growth hormone
BSA	Bovine serum albumin
C	Cytosine
CD	Cluster of differentiation
CD	Chemically defined
CDC	Complement-dependent cytotoxicity
cDNA	Complementary DNA
C_H	Constant region heavy chain
chim	Chimeric
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
CHYSEL	Cis-acting hydrolase element
C_L	Constant region light chain
CMV5	Cytomegalovirus 5
CSF	Colony-stimulating factor
dFBS	Dialyzed Fetal bovine serum
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia
eGFP	Enhanced GFP
EJC	Exon junction complex
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
et al	et alii

F2A	2A peptide from FMDV
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcR	Fc receptor
FDA	Food and Drug Administration
FGA	α-Fibrinogen
FITC	Fluorescein isothiocyanate
FMDV	Foot-and-mouth disease virus
FoxP3	Forkhead box P3
FSC	Forward scatter
Fuc	Fucose
G	Gram
G	Guanine
GAG	Glycosaminoglycan
Gal	Galactose
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GlcNAc	N-acetylglucosamine
GnTIII	N-acetylglucosaminyltransferase-III
GOI	Gene of interest
GS	Glutamine synthetase
GSA	Glutamic semi-aldehyde
h	Hour
HC	Heavy chain
HEK	Human embryonic kidney
HER2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
HT	Hypoxanthine and thymidine
hTGF-β1	Human transforming growth factor beta 1
Hum	Human
Hygro	Hygromycin B
hygroR	Hygromycin B resistance gene
ICC	Immunocytochemistry
IDUA	Iduronidase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IP	Immunoprecipitation
IRES	Internal ribosomal entry site
ITS	Insulin, transferrin, selenium
IVCD	Integral viable cell density

IVS	Intervening sequence
L	Liter
LAP	Latency associated peptide
LB	Lysogeny broth
LC	Light chain
LTBP1-4	Latent TGF-β-binding protein 1-4
M	Molar
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
Man	Mannose
max	Maximal
MCS	Multiple cloning site
MEM	Minimal essential medium
MFI	Mean fluorescence intensity
mg	Milligram
mGFP	Membrane-bound GFP
MGMT	Methylguanine-DNA-methyltransferase
Min	Minute
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MSX	Methionine sulphoximine
MTX	Methotrexate
N	Normal
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
Neo	Neomycin
neoR	Neomycin resistance gene
ng	Nanogram
OAT	Ornithine aminotransferase
OD	Optical Density
ORF	Open reading frame
P	Proline
P5C	Pyrroline-5-carboxylate
P5CDH	Pyrroline-5-carboxylate dehydrogenase
P5CR	Pyrroline-5-carboxylate reductase
P5CS	Pyrroline-5-carboxylate synthetase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pcd	pg/(cell*day)
pCMV/SV40	CMV/SV40 promoter
PCR	Polymerase chain reaction
pg	Picogram
polyA	Polyadenylation (signal)

POX	Proline oxidase
Pro-	Proline-auxotroph
Pro+	Proline-prototroph
R^2	Coefficient of determination
RIPA	Radioimmunoprecipitation assay
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
sec	Second
SSC	Sideward scatter
SV40	Simian virus 40
T	Thymine
T2A	2A peptide from *Thosea asigna* virus
TBS	Tris-buffered saline
T_H	T helper cells
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
Tregs	Regulatory T-cells
tRNA	Transfer RNA
U	Uracil
US	United States
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
V_H	Variable region heavy chain
V_L	Variable region light chain
WB	Western Blot
Zeo	Zeocin
zeoR	Zeocin resistance gene
γ-GK	γ-glutamyl kinase
γ-GP	γ-glutamyl phosphorylate
γ-GPR	γ-glutamyl phosphorylate reductase
γ-GSA	γ-glutamic semi-aldehyde
µg	Microgram
µL	Microliter
µM	Micromolar

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1 Abstract

The Chinese hamster ovary (CHO) cell line is currently the most important mammalian cell line for the production of therapeutic proteins. CHO cells are proline auxotroph due to deficiencies in both pathways to form proline either from glutamate or ornithine. The aim of this study was to use the proline auxotrophy of the CHO cell line as a selection system for the production of recombinant proteins.

The gene of the enzyme pyrroline-5-carboxylate synthetase (P5CS), which catalyzes the reaction of glutamate to pyrroline-5-carboxylate, was cloned into a dicistronic vector to couple the gene of interest (GOI) with the selection marker. Different CHO cell lines were analyzed in transfection experiments, growth tests in proline-free media and in Western Blots comparing isolated proline-prototroph cell lines to the auxotroph wildtype cells. These studies revealed that the CHO-S cell line is suited best for the proline selection system. Using the CHO-S cells, selection times and expression levels comparable to those of antibiotic selection with neomycin or hygromycin B could be reached. Expressing the green fluorescent protein (GFP), three different monoclonal antibodies (anti-CD303, anti-CD14, anti-Biotin) or the cytokine hTGF-β1, stable cell lines with producer rates of over 90% were generated in less than 20 days. For the expression of antibodies, a two-vector-strategy was chosen combining the proline selection system with a zeocin selection. Stable cell lines with titers up to 8 mg/L (static, adherent, in minimal medium) and clones expressing up to 140 mg/L (limiting dilution, orbital shake reactor, suspension, fed-batch process) were generated for the production of anti-CD303.

To further improve the proline selection system, a tricistronic vector allowing coexpression of fluorescent reporter proteins for the use of fluorescence- as well as magnetic-activated cell sorting (FACS and MACS) was developed. Using a stable cell line expressing anti-CD303 and coexpressing two cytosolic fluorescent proteins, clone isolation by FACS improved mean titer distribution of the isolated clones 1.9-fold when compared to a traditional limiting dilution. Membrane-bound fluorescent proteins allow a very fast and simple enrichment by MACS. Enrichment of stable cell lines producing anti-CD303 or hTGF-β1 revealed an initial 2.5- to 10-fold increase of titer, although these enhanced productivities decreased over the following culture time. Therefore, a combination of MACS enrichment of stable producer cell lines with clone isolation by FACS was proven to generate clones with similar productivities as clones isolated by two FACS subcloning steps, while dramatically improving timelines and manual amount of work. Clones producing up to 130 mg/L anti-CD303 antibody (orbital shake reactor, suspension, batch process) could be isolated by this combination of MACS and FACS. In addition to the demonstrated enrichment successes, proof for the correlation of intracellular hTGF-β1 and the cytosolic fluorescent protein levels was established by analyzing several FACS-isolated hTGF-β1-expressing clones.

The developed P5CS expression system enables a selection process without the need for any cytotoxic substances. It therefore offers an interesting alternative for the modification of parental production cell lines.

1.1 Zusammenfassung

Die *Chinese hamster ovary* (CHO) Zelllinie ist momentan die am häufigsten verwendete Mammaliazelllinie zur Produktion therapeutischer Proteine. Bereits seit der Isolation dieser Zelllinie ist bekannt, dass diese nicht in der Lage ist selbstständig Prolin aus den Vorläufern Glutamat oder Ornithin zu synthetisieren. Das Ziel dieser Arbeit war diese Prolinauxotrophie als Selektionssystem für die Produktion rekombinanter Proteine in CHO-Zellen zu nutzen.

Das Gen des Enzyms Pyrrolin-5-Carboxylat-Synthetase (P5CS), welches die Reaktion von Glutamat zu Pyrrolin-5-Carboxylat katalysiert, wurde in einen dizistronischen Expressionsvektor kloniert, um eine Kopplung des Gens von Interesse und des Selektionsmarkers zu erreichen. Verschiedene CHO Zelllinien wurden in erste Transfektionsstudien, Wachstumsversuche in prolinfreiem Medium sowie Western Blot-Analysen der prolinprototrophen Zelllinien im Vergleich zu den prolin-auxotrophen parentalen Zellen verglichen. Diese Studien ergaben, dass sich die CHO-S-Zelllinie am besten für ein prolinbasiertes Selektions-system eignet. Mit dieser CHO-S-Zelllinie können sowohl Selektionszeiten als auch Expressionslevels vergleichbar zu Antibiotikaselektionssystemen mit Neomycin oder Hygromycin B erreicht werden. Stabile Zelllinien, die das grün-fluoreszierende Protein GFP, drei verschiedene Antikörper (anti-CD303, anti-CD14, anti-Biotin) oder das Zytokin hTGF-β1 exprimieren, konnten in weniger als 20 Tagen mit Produzentenraten von über 90% generiert werden. Für die Expression der Antikörper wurde eine Zwei-Vektor-Strategie mit einer Kombination aus P5CS- und Zeocin-Selektion verwendet. Für die anti-CD303-Produktion konnten so stabile Zelllinien mit Titern von 8 mg/L (statisch, adhärent, in Minimalmedium) und Klone mit Titern von bis zu 140 mg/L (*limiting dilution*, Schüttelinkubator, Suspension, *fed-batch*) isoliert werden.

Um das neu etablierte Prolinselektionssystem weiter zu optimieren, wurde ein tricistronischer Expressionsvektor entwickelt, der die Koexpression von fluoreszierenden Reporterproteinen erlaubt und sowohl für magnetische als auch durchflusszytometrische Zellsortierung (MACS und FACS) genutzt werden kann. Im Vergleich zu einer traditionellen *limiting dilution* konnten aus einer anti-CD303-exprimierenden Zelllinie mit Koexpression zytoso-lischer Fluoreszenzproteine mittels FACS Klone mit einer 1,9-fach höheren durchschnittlichen Produktivität isoliert werden. Membrangebundene Fluoreszenzproteine können außerdem für eine schnelle Anreicherung mittels MACS genutzt werden. Sowohl eine anti-CD303- als auch eine hTGF-β1-exprimierende Zelllinie konnte um Faktor 2,5 bis 10 angereichert werden, wobei die Produktivität in der nachfolgenden Zeit in Kultur wieder leicht absank. Daher ist eine Kombination aus einer MACS-Anreicherung von stabilen Zelllinien mit einer anschließenden Klonisolation mittels FACS optimal, um sowohl Zeit- als

auch Arbeitsaufwand zu reduzieren. Desweiteren können mit dieser Kombination ähnlich produzierende Klone generiert werden wie mit zwei aufeinanderfolgenden FACS-basierten Klonisolationen. Klone mit einer Produktion von 130 mg/L des anti-CD303-Antikörpers (Schüttelinkubator, Suspension, *batch*) konnten mit dieser Kombination aus MACS und FACS isoliert werden. Bei der hTGF-β1 Produktion wurde zusätzlich eine Korrelation von intrazellulärem hTGF-β1 und dem zytosolischen Fluoreszenzprotein in FACS-isolierten hTGF-β1 exprimierenden Klonen nachgewiesen.

Das entwickelte P5CS-Expressionssystem erlaubt eine Selektion völlig ohne zytotoxische Substanzen. Daher bietet es eine interessante Alternative für die Modifikation von parentalen Produktionszelllinien.

2 Introduction

2.1 Recombinant protein production in CHO cells

Recombinant proteins play an essential role in research, diagnostics and clinical therapy. Due to their ability of correct folding, assembly, glycosylation and secretion (Wurm 2004, Khan 2008) about 60-70 % of the clinically approved recombinant proteins are produced in mammalian cell lines. Chinese hamster ovary (CHO) cell lines, isolated from a female inbred Chinese hamster (*Cricetulus griseus*) by Tjio and Puck (Tjio and Puck, 1958), are the most commonly used mammalian cell lines. Already Puck et al (1958) described the remarkable robustness of CHO cells, which together with the ease of transfection and adaptation to serum-free media, growth in high cell densities, high production levels and by now good characterization makes them the mostly used mammalian cell line (Beatson et al, 2011). Recently, Hammond et al (2012) published an online resource making the CHO genome publicly available[1]. In addition, under suitable production conditions, CHO cells are able to produce recombinant proteins with major N-linked glycoforms identical to those present in human proteins (Jefferis, 2005): Similar to mouse cells, the four major glycoforms G0, G1 (1-3), G1 (1-6) and G2 (Figure 1) occur in CHO produced N-glycoproteins (Wacker et al, 2011).

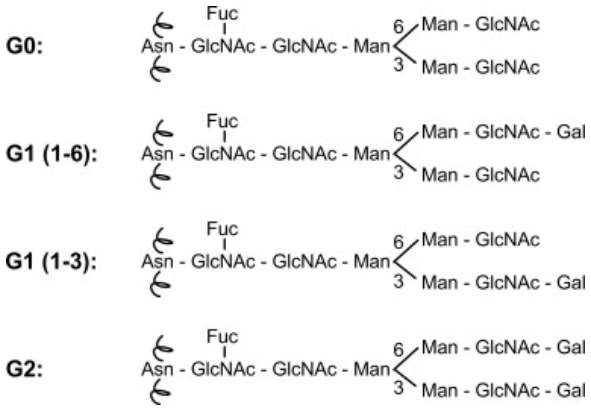

Figure 1: Structure of major N-linked glycoforms

Asn= asparagine, GlcNAc= N-acetylglucosamine, Fuc= fucose, Man= mannose, Gal= galactose (Wacker et al, 2011)

Different from human cells, CHO cells do not express N-acetylglucosaminyltransferase-III (GnTIII), an enzyme that mediates the transfer of bisecting N-acetylglucosamine (GlcNAc). Additionally, CHO cell derived antibodies contain α2,3-linked sialic acid residues, while human IgG mainly contain α2,6-linked sialic acid residues. But since α2,3-linked sialic acid residues have been reported in human IgGs, they do not seem to have immunogenic capacities. Both, the amount of bisecting N-acetylglucosamine as well as that of sialylated oligosaccharides is very small (<5-10% of glycans). In mouse produced antibodies contain

[1] http://www.chogenome.org/index.html, 25.10.2012

α1,3-linked galactose and N-glycolylneuraminic acid. Both epitopes are known to be immunogenic to humans and not to occur in hamster cells (Raju, 2003), making CHO cell lines more suitable for therapeutic protein production. In addition to these variations in N-linked glycosylation, there are several differences in O-glycosylation of human and CHO cell derived glycoproteins (Raju, 2003).

Generating production cell lines expressing high levels of recombinant proteins is a time consuming, labor-intensive process. Several different aspects, such as selecting a suitable CHO cell line, an optimized expression vector, an efficient transfection protocol and selection system as well as a fast and potent identification and isolation of high-producing clones, have to be considered. Following cell line development, a production process optimized for the selected clone has to be established. Depending on the used expression system, the time frame needed for identifying a suitable clone alone varies between over 6 month using a dihydrofolate reductase (DHFR) expression system and at least 3 months for the glutamine synthetase (GS) Gene Expression System™ (Butler, 2005), while still requiring clone stability and production tests as well as first process optimization. This results in cell line development times of 6 months to over a year (Wurm, 2004), as schematically shown in Figure 2 for a traditional amplified selection system (Haines, 2009). Therefore there are ongoing efforts to improve cell development using new technologies to reduce timelines and reach a more robust and faster process (Birch and Racher, 2006).

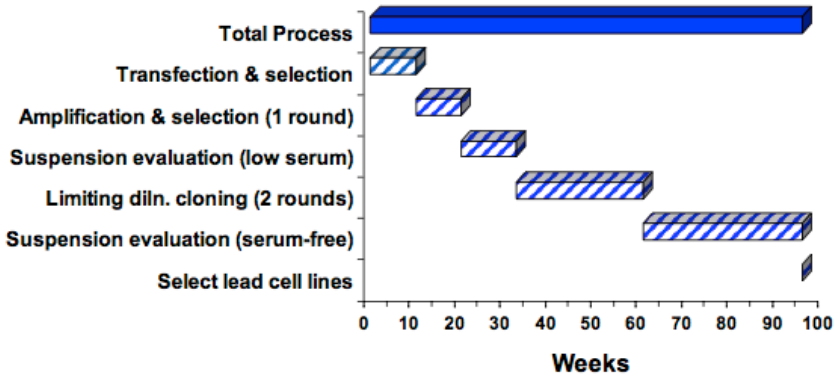

Figure 2: Schematic overview of the cell line development timeline
Overview of cell line development process using a traditional amplified system with clone isolation by limiting dilution (Haines, 2009)

2.1.1 Selection systems for mammalian cells

Several different selection systems either based on antibiotic selection or on metabolic selection are known. Common antibiotic selection systems include resistance to neomycin, hygromycin B, puromycin or zeocin (Wurm, 2004). Using these selection systems, the resistance gene is integrated into the expression vector and the corresponding antibiotic is added into the culture medium. Only positively transfected cells expressing the resistance

gene are able to survive the applied selection pressure. In selection systems based on metabolic enzymes on the other hand, the gene encoding a metabolic enzyme is integrated into the expression vector and positively transfected cells are able to survive selection in a medium missing the corresponding nutrient. Currently available and commonly used expression systems based on selection with metabolic enzymes are the DHFR expression system (Haynes and Weissmann, 1983), which is based on the selection with the enzyme dihydrofolate reductase in a medium lacking hypoxanthine and thymidine, and the GS Gene Expression System™ (Cockett et al, 1990), which is based on the selection with the enzyme glutamine synthetase (GS) in a glutamine-free medium. For the DHFR selection system, special cell lines with a deficiency in DHFR activity, such as the CHO DG44 or CHO DXB11, are required. The DHFR enzyme catalyzes the reduction reaction from dihydrofolate acid to tetrahydrofolic acid (coenzyme F), which is an essential methyl donor for the amino acid and especially the nucleic acid (thymine and purine) metabolism. The DHFR inhibitor methotrexate (MTX), which acts as a folic acid antagonist, can be used for amplification by stepwise increase of inhibitor concentration and selection of resistant cells. This time-consuming MTX amplification facilitates high copy numbers and therefore increases recombinant protein production, but implicates a high cytotoxicity (Kaufman et al, 1985) and genetic instability of the production cell lines (Kim et al, 1998 A). In addition, MTX transporter mutations are known to lead to resistant phenotypes (Assaraf and Schimke, 1987). For the GS selection no special cell lines are required, although CHO cells endogenously express the glutamine synthetase. Using the toxic drug methionine sulphoximine (MSX), a glutamine synthetase inhibitor, facilitates selection and may be used for amplification. Recently GS-knock out cell lines generated by zinc-finger-nucleases have been described (Liu et al, 2010) to increase the selection stringency and to lead to an elevated recombinant protein production (Fan et al, 2012).

2.1.2 Different CHO cell lines

Several different CHO cell lines have been derived from the originally isolated CHO cell line. Early subclones were mainly generated to study the genetics and metabolism of mammalian cells. More recently, since CHO cells have evolved into the major mammalian cell line for recombinant protein production, a number of special production strains have been isolated. The CHO-K1 cell line is a subclone derived directly from the first isolated CHO line (Kao and Puck, 1969). It only possesses 20 chromosomes compared to 22 in the normal Chinese hamster (Taylor and Hanna, 1982). The CHO-S cell line, a separate subclone of the parental CHO cell, is characterized as less anchor-dependent compared to the CHO-K1 cell line (Kurano et al, 1990). In addition, the CHO-S cells are aneuploid (D´Anna et al, 1996) containing 21 chromosomes (Taylor and Hanna, 1982). Invitrogen recloned this CHO-S cell line by limiting dilution to obtain a cell line (FreeStyle™ CHO-S) further optimized for

growth in serum-free conditions and with improved transfection efficiencies[2]. Several different subclones with mutated phenotypes with regard to metabolic markers have been isolated from these parental cell lines; of importance for recombinant protein expression are the DHFR deficient cell lines CHO DXB11 and CHO DG44. Urlaub and Chasin (1980) isolated the CHO-K1 subclone CHO DXB11 (DUXB11) by chemical and radioactive mutagenesis. This subclone has a deletion in one of the DHFR alleles and a missense mutation in the other. Therefore reversion to a DHFR prototroph phenotype can be observed; reversion rates range between 0.001% described by Urlaub and Chasin (1980) and 0.31% in serum-free suspension media described by Sinacore et al (1996). Three years later, the MTX-resistant mutant CHO MtxRIII, isolated from the proline-dependent CHO-pro3 strain, was mutagenized by ionizing radiation to yield the CHO DG44, a cell line with deletion of both DHFR alleles (Urlaub et al, 1983).

2.1.3 Vector design

The gene of interest (GOI) encoding for the recombinant protein and the selection marker gene are inserted into the host cell by expression vectors. These expression vectors are mainly plasmids consisting of circular double-stranded DNA. In addition to the GOI and the selection marker gene, vectors encode for a bacterial replication site and a bacterial selection marker to facilitate plasmid amplification and isolation from bacteria. For expression in mammalian cells, the expression vector contains a suitable promoter for transcription initiation of the following gene. To archive high expression of the GOI, a strong promoter such as the cytomegalovirus 5 (CMV5) promoter is needed. The CMV5 promoter is an improved CMV promoter consisting of the human CMV immediate early promoter and enhancer. Additionally this promoter contains the tripartite leader (Ad-tpl) and a splice site from the human adenovirus 5 major late promoter. Compared to the non-optimized CMV promoter, the CMV5 promoter enables expression of about 5-12 –fold higher recombinant protein levels in HEK 293 cells (Massie et al, 1998). In a traditional expression vector, each gene is transcribed individually and is therefore controlled by its own promoter (Figure 3). For the expression of the selection marker gene, a less efficient promoter than the CMV5 promoter, such as the *simian virus 40* (SV40) early promoter, is used. The disadvantage of this vector design is the independent expression of GOI and selection marker gene allowing cells, which only express the selection marker gene, to survive in the selection medium. This expression of selection marker but no GOI might be either due to integration of only the selection marker expression cassette or subsequent loss or down-regulation of the integrated GOI (Pu et al, 1998). These non-producers might even have a growth advantage due to a reduced expression burden compared to producing cells and may overgrow the latter in a mixed population.

[2] http://de-de.invitrogen.com/site/de/de/home.html, 25.10.2012

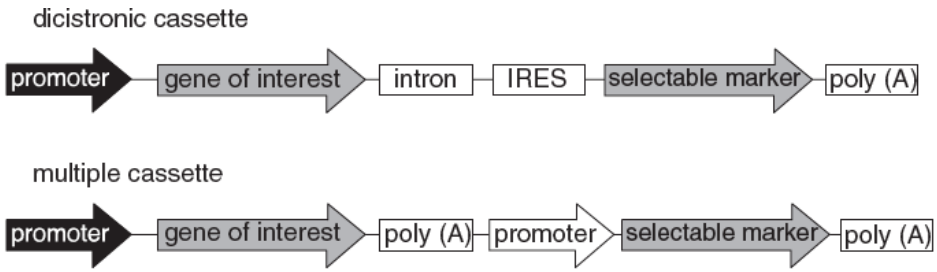

Figure 3: **Comparison of different vector designs**
Two different vector designs used for the expression of recombinant proteins. In a multiple cassette, the gene of interest and the selection marker are encoded by a promoter and polyA signal for each gene. In a dicistronic cassette, the gene of interest and the selection marker are controlled by a single promoter and polyA signal due to a coupling element such as an IRES element (Chen et al, 1998).

This effect can be avoided by coupling of GOI and selection marker gene in a dicistronic expression cassette (Figure 3). This might be realized by integrating an internal ribosomal entry site (IRES) between GOI and selection marker gene. IRES elements were first identified in the polio virus (*Human enterovirus C*) and *Encephalomyocarditis virus* (EMCV) but have also been identified in several other viruses and eukaryotic organisms ranging from yeast to mammals. The 80 to 500 nucleotide long element allows a cap-independent translation of the mRNA. The gene cloned downstream of the IRES element is translated less efficiently (10-50%) compared to the upstream cap-dependent translation (Szymczak and Vignali, 2005). For expression of recombinant proteins, the coupling of GOI and selection maker gene has some major advantages: First, since GOI and selection marker gene are encoded on one common mRNA, cells are not able to exclusively express the selection marker gene. Thereby, the frequency of non-producers is massively reduced. Second, the reduced IRES-dependent expression of the selection marker compared to the cap-dependent expression of GOI ensures higher levels of GOI expression in order to reach selection marker amounts sufficient to survive the applied selection (Pu et al, 1998).
Another element suitable to couple different genes is a 2A peptide sequence. These about 18-22 amino acid long sequences can be found in picornaviruses like foot-and-mouth disease virus (FMDV) and EMCV, insect viruses and Type C rotaviruses (*Rotavirus C*). The 2A peptide sequence contains a highly conserved consensus motif at the C-terminus (D-V/I-E-X-S-N-P-G*P), which mediates the "cleavage" between the C-terminal glycine and the N-terminal proline. This "cleavage" does not require any additional factors like proteases and was first thought to be an autoproteolytic event, but recently the "cleavage" has been proposed to be a ribosomal-skip mechanism (Figure 4) and 2A peptides are referred to as CHYSELs (cis-acting hydrolase elements) (Szymczak and Vignali, 2005).

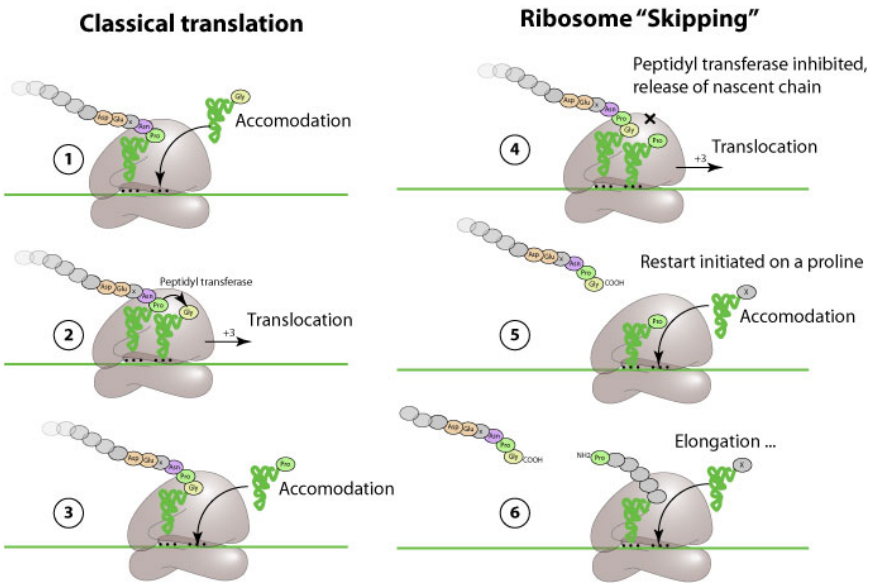

Figure 4: Ribosomal-skip mechanism at 2A peptide

Ribosome skipping at the 2A peptide sequence: The 2A peptide disrupts translation and impairs normal peptide bond formation resulting in translation of two separate proteins from one open reading frame (ORF) [3].

The primary translated pre-mRNA is processed into a mature mRNA and transported into the cytoplasm for translation. For these processes, several elements are necessary on an expression vector to initiate processing and transport. Shortly after transcription, a modified guanine nucleotide is added to the 5´ end of the mRNA resulting in the cap-structure, which is necessary for cap-dependent translation. This process does not require any additional sequences. To protect the mRNA from degradation, the expression vector contains a polyadenylation signal (PolyA), which leads to addition of a tail of multiple adenosine monophosphates at the 3´ end. Azzoni et al (2007) compared the bovine growth hormone (BGH), the SV40 and a synthetic PolyA signal in CHO. Using the BGH PolyA signal led to the highest numbers of transfected cells and highest GOI expression levels making it a suitable PolyA signal for an expression vector for high level recombinant protein expression. Eukaryotic protein-coding genes often contain introns, which are non-coding sequences between the protein-coding sequences (exons). These introns are removed from the pre-mRNA by splicing according to donor and acceptor splice sites surrounding the intron sequence. The mRNA splicing and its export into the cytoplasm are closely connected. The exon junction complex (EJC) formed by different proteins at the junction of two exons is a platform for the binding of several export factors. This splicing memory ensures that only mature mRNAs consisting of exons are exported (Knippers, 2006). In expression vectors for recombinant protein expression, genes are mainly encoded as cDNA. Different from genomic DNA they do not contain introns. Since mRNA splicing and export are closely connected, the lack of introns might lead to reduced mRNA export (Huang and Gorman, 1990; Buchmann and Berg, 1988). Therefore, most expression vectors contain at least one

[3] http://viralzone.expasy.org/all_by_protein/914.html, 25.10.2012

intron (Walls et al, 1993). Positive, but sequence-dependent and cell line-dependent influences of these added introns on expressed recombinant protein levels have been observed (Huang and Gorman, 1990; Mariati et al, 2010).

2.1.4 Clone isolation

For high-level recombinant protein production not only an optimized vector and selection system is necessary, but also the process of clone isolation is critical. After transfection, only about 0.1% of the cells stably integrate the foreign DNA into their genome (Gray, 1997). Although the applied selection pressure enriches the number of producing cells, the remaining stable producer cell line is still a mixture of cells with different integration sites, expression levels and growth properties. Identifying clones with extraordinarily high production capacities and stability as well as growth characteristics suitable for the production process is a labor- and time-consuming process. The most simple method for clone isolation is limiting dilution to concentrations that will likely dispense one or less than one cell per culture well. Each up-growing clone has to be screened for its production and growth capacities. While automation of this process can enhance the number of screened clones, the number remains a limiting factor (Chartrain and Chu, 2008). Therefore, different methods have been developed allowing a faster identification and isolation of clones according to their production capacities.

For isolation of high-producing clones, two sorting methods come into consideration. The fluorescence activated cell sorting (FACS) is of special importance for the isolation of the identified high-producing clones as it allows isolation of cells according to their fluorescence as single cells or in a bulk. To obtain high purities, lower flow rates of about 1,000 to 3,000 cells per second are suitable allowing the screening of up to $1x10^7$ cells per hour. Another potent method for cell enrichment is the magnetic activated cell sorting (MACS), although it has only been described for isolation of high-producing stable cell lines in few publications. For example Hoch (2010) developed an "antibody CHO capture system" and Carroll and Al-Rubeai (2005) describe a MACS-based high-producer enrichment protocol using affinity capture surface display (ACSD). The MACSelect™ system (Miltenyi Biotec) allows isolation of transiently transfected cells by MACS using different cell surface markers, but does not include a selection system. For magnetic separation, cells expressing a surface marker are labeled directly or indirectly with antibodies conjugated to superparamagnetic particles. Ferromagnetic columns are placed in a magnetic field amplifying the magnetic field 10,000-fold. Labeled cells are applied onto the column and non-magnetically cells flow through the column. Magnetically labeled cells are retained and can be eluted in the following step (Miltenyi et al, 1990) (Figure 5). Isolation of positive cells from a starting population of up to $2x10^{10}$ cells is possible in one isolation step in less than 2 hours (Philipps, 2004).

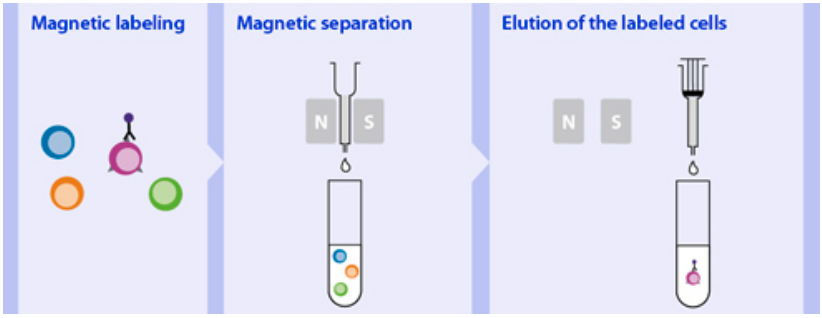

Figure 5: Schematic overview of a positive enrichment with MACS
Target cells expressing a certain surface marker are labeled with a magnetic bead-coupled antibody and applied to a column placed in a magnetic field. The unlabeled cells pass through while the magnetically labeled cells are retained within the column. After a washing step, the column is removed from the separator, and the magnetically labeled cells can be eluted from the column [4].

To isolate high-producing cells one first needs to identify these cells. Several methods to identify high-producing cells are based on the detection of the secreted recombinant protein: Holmes and Al-Rubeai (1999) used an immobilized affinity surface display matrix that specifically bound the secreted target protein, which was then detected by a fluorescently labeled antibody and the brightest fluorescent cells were finally isolated by FACS. With this method it is possible to screen up to $5x10^6$ cell in about 4 hours. This method of capturing the secreted recombinant antibody in an antibody-capture matrix can also be used in combination with MACS instead of FACS described by Carroll and Al-Rubeai (2005) and Hoch (2010). A similar approach was used by Caron et al (2009), where cells were seeded in a semi-solid medium ensuring clonal colonies and trapping secreted protein in the surroundings of the colonies. A FITC-labeled secondary antibody specific for the secreted recombinant protein was used for staining. Large and bright colonies indicating good growth and productivity were isolated by automated or manual clone picking. Secreted recombinant proteins can also be detected without a capture matrix using a "cold capture assay". According to Brezinsky et al (2003), secreted recombinant antibodies stay transiently attached to the cell membrane at low temperatures and can be stained using a labeled antibody.
The second possibility to identify high-producing clones is via a modified vector coexpressing reporter proteins, such as fluorescent proteins or surface proteins. These surface proteins are usable for antibody staining, followed by clone isolation by FACS or fluorescence plate readers. Different vector configurations, reporter proteins and isolation strategies have been described: Meng et al (2000) reported the coexpression of GFP in a separate transcription unit. In contrast Mancia et al (2004) prefered the GFP coupled to the GOI via an IRES to improve correlation of GOI and GFP expression. Sleiman et al (2008) used two IRES-coupled reporter proteins for each heavy and light chain of a recombinant antibody. Like Meng et al (2000) and Mancia et al (2004) Sleiman et al (2008) used FACS to

[4] http://www.miltenyibiotec.com/en/NN_1146_Basic_principle.aspx, 25.10.2012

isolate the brightest cells. An IRES-coupled GFP can also be used for identification of high-producers by fluorescence plate reader (Freimark et al, 2010). Instead of fluorescent proteins, IRES-linked reporter genes encoding surface markers such as CD2 or CD4 might be used in a FACS approach (DeMaria et al, 2007; Liu et al, 2000). A different vector design allowing coupled expression of GOI and reporter protein was described by Cairns et al (2011). In detail, non-AUG start codons to initiate reporter protein (CD52) expression with reduced efficiency upstream of the GOI were used in combination with FACS. A very interesting vector design was described in a patent application by Jostock et al (US 2001/0281751 A1) using a "leaky" stop, which terminates the recombinant protein followed by a membrane anchor in frame. A certain amount of ribosomes reads over the first "leaky" stop and thereby the recombinant protein is expressed as a membrane-bound form and can easily be used for detection. Another possible vector design includes the fusion of reporter and selection marker protein, as described by Bennett et al (1998) for a GFP-zeocin selection marker fusion and by Bailey et al (2002) for a GFP-metallothionein fusion, which confers heavy metal resistance.

In addition to capturing secreted recombinant proteins or coexpression of fluorescent proteins by novel vector designs, Kober et al (2012) described a modified CHO cell line allowing identification of high-producers. Overexpression of recombinant proteins triggers endoplasmatic reticulum (ER) stress response. A generated CHO DG44 cell line with stable integration of GFP controlled by the ER stress sensitive glucose-regulated protein 78 kDa (GRP78) promoter allows FACS isolation of clones. These clones show a relation between GFP ER stress signal and recombinant antibody expression.

2.1.5 Recombinant proteins

Complex glycosylated proteins require mammalian expression systems to facilitate correct folding, assembly, glycosylation and secretion of the proteins (Wurm, 2004; Khan and Schroder, 2008). The first recombinant protein produced in CHO cells that gained FDA-approval for therapeutic use in humans was the human tissue plasminogen activator (tPA) in 1987 (Kozlowski, 2007). By 2010 recombinant proteins such as hormones, growth factors or antibodies comprised most of the over 200 approved biopharmaceuticals (Walsh, 2010) with CHO cell lines as the predominantly used host cell line (Costa et al, 2009). But recombinant proteins produced by mammalian cells are not only used for therapeutic applications in humans. The applications range from veterinary pharmaceuticals to research and diagnostic applications.

2.1.5.1 Monoclonal antibodies

Antibodies, also called immunoglobulins, are heterodimeric proteins composed of two heavy chains of about 50-70 kDa and two light chains of about 25 kDa. Non-covalent and covalent

bonds connect the chains. The two heavy chains are connected to each other and to the light chains by disulfide bonds. There are five different types of heavy chains (μ, δ, γ, α and ε), resulting in the different immunoglobulin subclasses (IgM, IgD, IgG, IgA and IgE) (Holländer, 2006). Most therapeutically used antibodies belong to the IgG subclass (Chartrain and Chu, 2008) (Figure 6). The constant region of IgG molecules contains one conserved glycosylation site in the Fc region resulting in a N-linked biantennary structure at Asn 297 (Wright and Morrison 1997). The glycosylation pattern plays a critical role for the effector function and immune clearance (Wright and Morrison, 1997). Deglycosylated IgGs have been reported to inefficiently mediate Fc receptor binding therefore being unable to trigger antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Abés and Teillaud 2010). Similar hypogalactosylation and monosialylation of IgG lead to poor activity in ADCC, while blockage of the processing of the oligomannose intermediate generates IgG1 unable of complement-dependent cytotoxicity (CDC). In addition, the presence of oligomannose (Abès and Teillaud, 2010) or terminal (α1→3) galactose expressed by murine cells (Wright and Morrison, 1997) leads to a rapid clearance of the antibody.

For the light chain, two different types are known: κ- and λ-chains. But no functional difference has been observed. Heavy (H) and light (L) chain are both formed by a constant (C) and a variable (V) region: The variable regions V_L and V_H mediate the ability of recognition of specific antigens; the constant regions C_L and three to four C_H regions, depending on the heavy chain subtype, mediate effector functions (Holländer, 2006) (Figure 6).

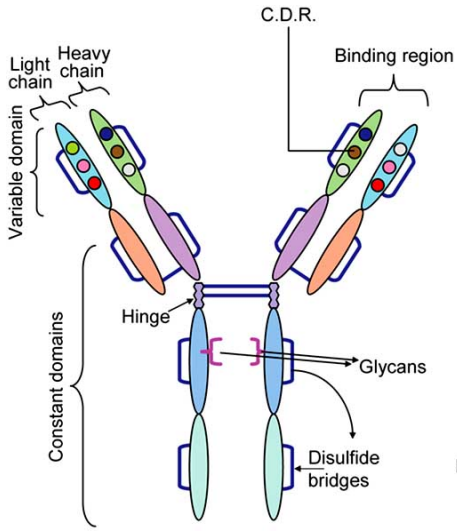

Figure 6: Schematic structure of IgG antibodies

IgG molecules consist of two heavy and two light chains connected by disulfide bonds. They form a variable and constant domain connected by the hinge region. Each heavy chain contains a conserved glycosylation site at Asn 297 (Chartrain and Chu, 2008).

Monoclonal antibodies have become increasingly important for research and diagnostics as well as therapeutic applications. Their ability to specifically bind a target molecule is used in several research and diagnostic methods including Enzyme-Linked Immunosorbent Assay (ELISA) (Engvall and Perlmann, 1971), Enzyme Linked Immunospot (ELISPOT) (Czerkinsky et al, 1983), Immunoblot (Renart et al, 1979), Immunoprecipitation (IP) (Bonifacino et al, 2001), Immunohistochemistry (IHC)/Immunocytochemistry (ICC) (Coons et al, 1942) and flow cytometry (Herzenberg and Sweet, 1976). Antibodies are also used for cell enrichment strategies such as FACS (Herzenberg and Sweet, 1976) and MACS (Miltenyi et al, 1990).
In addition, recombinant antibodies are used for therapeutic applications. They constitute more than 30% of the total biopharmaceutical production and are the major part of proteins currently tested in clinical trials (Rodriges et al, 2010). The two major application fields of therapeutic monoclonal antibodies are cancer and autoimmune diseases. The "big five" therapeutic antibodies representing about 78% of the total antibody sales in the US reflect this focus: Infliximab (Remicade®) and adalimumab (Humira®) targeting the tumor necrosis factor alpha (TNF-α) are used to treat the autoimmune diseases rheumatic arthritis, Crohn's disease and plaque psoriasis. Trastuzumab (Herceptin®) targets the human epidermal growth factor receptor 2 (HER2) and is used as well as bevacizumab (Avastin®) targeting the vascular endothelial growth factor A (VEGFA) to treat several types of cancer including colorectal, lung, breast, glioblastoma, kidney and ovarian. The fifth best selling antibody rituximab (Rituxan®/Mabthera®) targets CD20 and is used against both rheumatoid arthritis and non-Hodgkin's lymphoma (Leavy, 2010).

2.1.5.2 Cytokines

Cytokines are cell-signaling molecules, which are released by various cells in the body as answer to an activating stimulus (Holländer, 2006). They are small (about 25 kDa), glycosylated proteins, which require an expression system with suitable glycosylation patterns (Ramani and Kondaiah, 1998). Cytokines can be divided into several families such as interferons (IFN), interleukins (IL), tumor necrosis factors (TNF), colony-stimulating factors (CSF) and transforming growth factors (TGF) (Holländer, 2006).
The TGF family consists of homodimeric proteins with a broad range of biological activities. For TGF-β, three isoforms are known in mammals: β1, β2 and β3 (Bourdrel et al, 1993). First isolated from human platelets, TGF-β1 is best characterized of this group (Gentry et al, 1987) and is expressed by nearly all cell types in low amounts. Interestingly, TGF-β1 is a highly conserved protein with amino acid homologies of 100% between porcine, canine and bovine and 99% homology between the mouse, rat and equine orthologs (Beatson et al, 2011). The human and the murine forms only differ in one amino acid (Gentry et al, 1987). Human TGF-β1 is expressed as a 390 amino acid precursor molecule containing a 29 amino acid leader

sequence. Immature TGF-β1 is activated by proteolysis to the mature TGF-β1. When matured, TGF-β1 contains only the 112 C-terminal amino acids of the precursor and forms a homodimer (25 kDa) of two mature TGF-β1 molecules bound by non-covalent interactions. The mature homodimer still remains in a latent form due to the binding of the latency associated peptide (LAP) forming the small latency complex. Alternatively, the homodimer bound to LAP can additionally bind the latent TGF-β-binding protein 1-4 (LTBP1-4) forming the large latency complex. This latent form can be activated by several mechanisms: *In vitro* by acid or heat, *in vivo* also by acid (pH stress), thrombospondin interaction, protease cleavage or mild mechanical stress. Active TGF-β1 inhibits growth of cells expressing the TGF-βRII receptor on their surface by up-regulation of p15 and p21 leading to cell cycle arrest. It therefore plays an important role in development, differentiation, wound healing, metastasis and angiogenesis (Beatson et al, 2011). TGF-β1-knockout mice show a severe inflammation response (Ramani and Kondaiah, 1998) demonstrating the importance of TGF-β1 for immunosuppression and immunotolerance. TGF-β1 is responsible for the induction of regulatory T-cells (Tregs), the class switch in B-cells from IgG to IgA production, the induction of tolerogenic antigen presenting cells and the inhibition of Th1 and Th2 T-cell development (Beatson et al, 2011). Recombinantly produced TGF-β1 is of interest for both research and clinical applications. The potent immunosuppressive effects of TGF-β1 make it an interesting candidate for treatment of autoimmune diseases such as multiple sclerosis (Mirshafiey and Mohsenzadegan, 2009). In research, TGF-β1 has several applications. For example, it can be utilized for the *in vitro* differentiation of naive $CD4^+$ T cells towards T_H17 cells (Ayyoub et al, 2012), the *in vitro* generation of $FoxP3^+$ inducible regulatory T cells (iTregs) (Horwitz et al, 2008) and stem cell differentiation models, since TGF-β1 regulates the stemness as well as various differentiation pathways (Puceat, 2007).

2.2 Proline metabolism

Proline is one of 22 natural genetically encoded proteinogenic amino acids. It is traditionally classified as nonessential in mammals, since there are two metabolic pathways available to synthesize proline either from glutamate or ornithine (Hu et al, 2008 B) as shown in Figure 7.

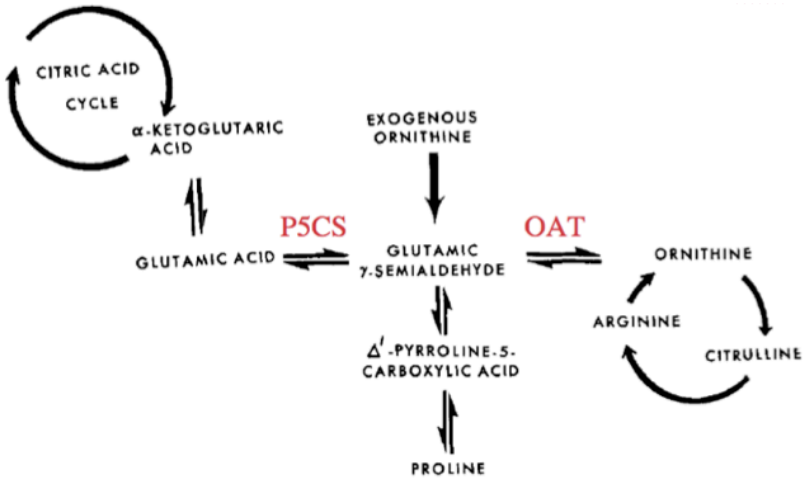

Figure 7: Metabolic pathways involved in proline synthesis

In mammals there are two proline-forming pathways, both joining at γ-glutamic semi-aldehyde (GSA). GSA can either be formed from glutamic acid linking proline synthesis to the citric acid cycle catalyzed by the enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) or from ornithine by the enzyme ornithine aminotransferase (OAT) linking proline synthesis to the urea cycle (modified after Kao and Puck, 1967).

The bifunctional ATP- and NAD(P)H-dependent enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) catalyzes the conversion of glutamate to γ-glutamic semi-aldehyde (GSA), which is in tautomeric equilibrium with Δ^1-pyrroline-5-carboxylate (P5C) (Figure 8). P5CS contains two enzymatic domains: The γ-glutamyl kinase (γ-GK) converts glutamate to γ-glutamyl phosphorylate (γ-GP), a labile and reactive intermediate. The γ-glutamyl phosphorylate reductase (γ-GPR) then enables the reaction to γ-glutamic semi-aldehyde (γ-GSA) (Hu et al, 2008 A). Two different P5CS isoforms generated by alternative splicing are known in multicellular eukaryotes. They differ only by a two amino acid insert at the N-terminus of the γ-GP site: P5CS.short is non-competitively inhibited by ornithine whereas P5CS.long is insensitive to ornithine. P5CS is a unidirectional enzyme. The reverse reaction from pyrroline-5-carboxylate to glutamate is catalyzed by pyrroline-5-carboxylate dehydrogenase (P5CDH). The key enzyme of the second proline-forming pathway is the ornithine-aminotransferase (OAT) catalyzing the reversible reaction from ornithine to GSA. Different from the enzyme P5CS, both reactions are catalyzed by the bidirectional OAT. Both P5CS and OAT are nuclear-encoded, but are localized in the mitochondria: P5CS in the inner membrane and OAT in the matrix (Hu et al, 2008 A). From pyrroline-5-carboxylate, which forms an equilibrium with γ-glutamic semi-aldehyde, synthesis to proline is catalyzed by the cytosolic enzyme pyrroline-5-carboxylate reductase (P5CR) (Figure 8). The opposite reaction from proline back to P5C is enabled by the inner-membrane mitochondrial enzyme proline oxidase (POX) (Hu et al, 2008 B).

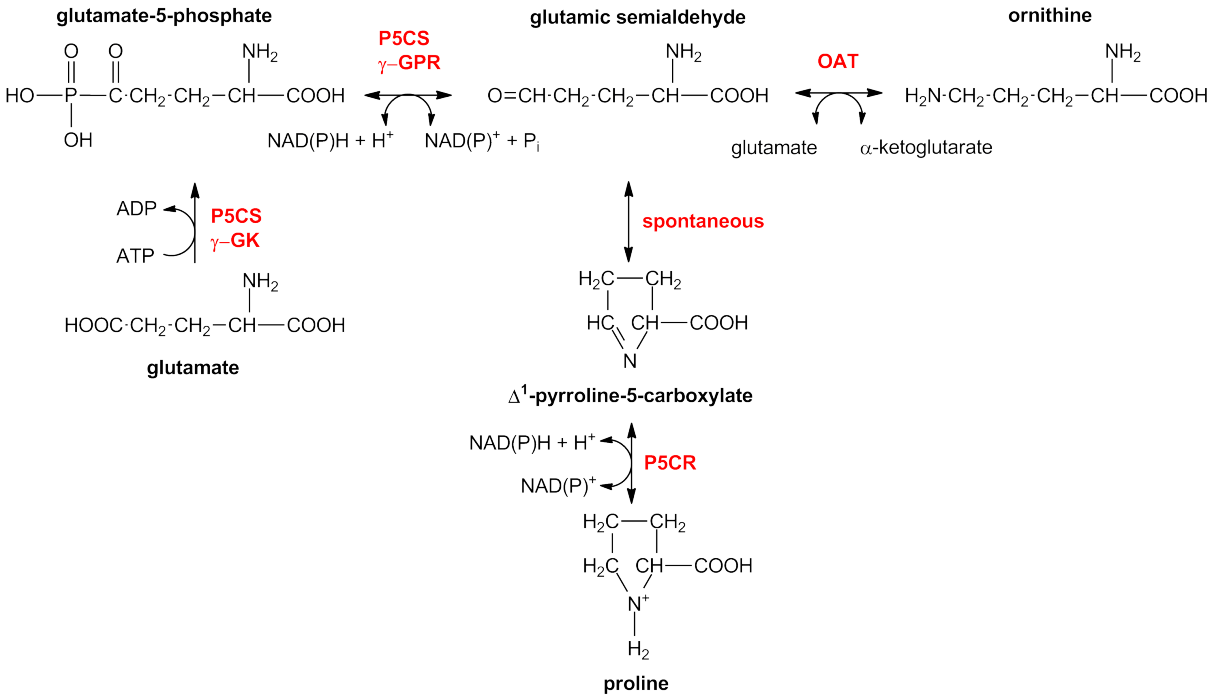

Figure 8: Proline forming pathways from glutamate and ornithine in detail

The bifunctional enzyme P5CS with kinase and dehydrogenase activity facilitates the conversion of glutamate to glutamic semialdehyde via the intermediate glutamate-5-phosphate. In addition, glutamic semialdehyde can be synthesized from ornithine catalyzed by the enzyme OAT. The common intermediate is in an equilibrium with the spontaneously formed Δ^1-pyrroline-5-carboxylate, which can then react to proline catalyzed by the enzyme P5CR.

The CHO cell line is known to be proline auxotroph. Kao and Puck (1967) analyzed the required proline concentration in media for growth and survival of the proline auxotroph parental CHO cell line and described the formation of proline prototroph cell lines from proline-free media. This proline auxotrophy is caused by a deficiency in both available proline catabolic pathways: Baich (1977) describes a deficiency of CHO-K1 cell in the OAT function and Smith et al (1980) were able to demonstrate the inability of CHO-K1 to form P5C from glutamate indicating a deficiency in the P5CS enzyme. Hu et al (1999) were able to demonstrate that both murine P5CS isoforms complement the proline auxotroph phenotype in CHO cells. After transfection of murine P5CS cDNA into a subclone of CHO-K1 cells known for deficiency in P5CS activity, they selected stable transfectants in a medium lacking proline and assayed P5CS activity on individual clones.

2.3 Aim of thesis

CHO cell lines are the major host cell lines for recombinant protein production for therapeutic as well as research applications due to their ability of post-translational modifications. Cell line development is currently one of the critical steps. Therefore, there are ongoing efforts to further improve not only the timeline of cell line development but also the development of novel robust selection and enrichment strategies.

The aim of this project is to establish an improved CHO expression system for the production of recombinant proteins. One aspect is the development and characterization of a novel selection system based on the proline metabolism. Furthermore, the project comprises the development of an improved process for the isolation of high-producers.

CHO cells are known to be proline auxotroph (Kao and Puck, 1967). But no selection system based on the proline metabolism has been described so far. In the first step, the proline metabolism of different CHO cell lines will be characterized by isolation of proline prototroph cell lines from proline-free medium and analysis of these different cell lines regarding P5CS and OAT sequence, protein expression and mRNA levels. Based on these results, a novel selection system using the enzyme P5CS as a selection marker should be established. This includes the construction of a suitable dicistronic expression vector as well as the optimization of transfection and selection protocols for a fluorescent protein. Once the novel expression system will be established, general applicability should be demonstrated by the expression of different recombinant proteins of interest for production processes including monoclonal antibodies and cytokines. To further analyze production capacities of the novel expression system based on P5CS, clones will be isolated and analyzed in batch and fed-batch shake flaks cultures.

In addition to a suitable selection system, the isolation of clones with extraordinarily high production capacities and growth characteristics suitable for the production process is a critical step during cell line development. In the second part of this project a tricistronic expression vector allowing coupled expression of GOI, selection marker and an additional reporter protein will be constructed and optimized making use of the novel P5CS selection system. GOI and reporter protein expression should correlate due to the coupled expression and will be revised. Finally, high-producer isolation by both FACS and MACS will be established and compared to traditional clone isolation by limiting dilution.

3 Results

3.1 Development of a novel selection system

3.1.1 Characterization of proline metabolism in CHO cells

3.1.1.1 Isolation of proline-prototroph CHO cell lines

To characterize their ability to grow in proline-free medium, CHO-K1 cells were seeded in a 24-well plate at $2x10^4$ cells per well in mimimum essential medium (MEM), supplemented with 10% dialyzed FBS (dFBS), glutamine without and with 40 mg/L proline. Cells of two wells were detached from the plate using trypsin and counted in a Neubauer chamber on a daily base. As shown in Figure 9 A, CHO-K1 cells cultivated in a medium containing proline grew to a maximal cell number of $5x10^5$ cell per 24-well within 5 days. In contrast, cells cultivated in proline-free medium had a period of over 10 days with no visible growth. Eventually, single colonies recovering to a proline-prototroph (Pro+) cell line started to grow up. This Pro+ cell line showed similar growth properties in proline-free medium as wildtype proline-auxotroph CHO-K1 cells in medium containing proline, as shown in Figure 9 B. From the number of seeded cells and the number of visibly formed colonies in proline-free medium, a reversion rate of 0.01 to 0.015% from a proline-auxotroph (Pro-) to a proline-prototroph (Pro+) phenotype for CHO-K1 cells could be calculated.

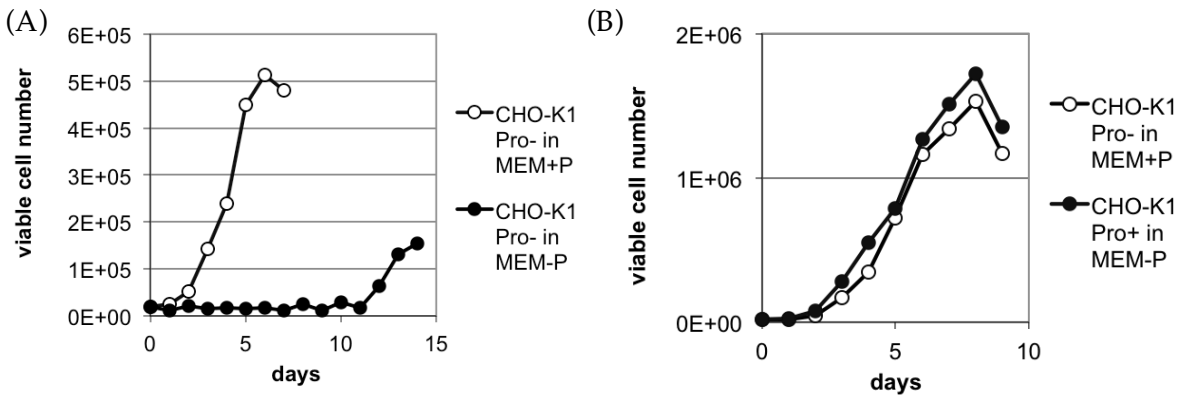

Figure 9: Isolation of proline-prototroph CHO-K1 cell lines

(A) $5x10^5$ CHO-K1 proline-auxotroph (Pro-) cells per 24-well were cultivated in MEM supplemented with 10% dialyzed FBS with proline (Pro- in MEM+P) and without proline (Pro- in MEM-P). (B) $5x10^4$ CHO-K1 Pro+ cells were in proline-free medium and wildtype CHO-K1 Pro- cells in proline-supplemented medium.

3.1.1.2 Expression of P5CS and OAT in different CHO cell lines

As described for the CHO-K1 cell line, Pro+ cell lines able to survive and grow in proline-free medium (MEM supplemented with glutamine and 10% dialyzed FBS) were isolated from CHO-S and CHO DG44 cells in the same manner. Western Blot analysis (Figure 10) of P5CS protein levels revealed no detectable P5CS expression in all Pro- parental cell lines. In

the Pro+ cell lines, however, P5CS expression could be detected although P5CS levels differed between the different Pro+ cell lines. The CHO-S Pro+ cell line did not express detectable amounts of P5CS; the CHO DG44 Pro+ expressed P5CS at barely detectable levels, whereas the CHO-K1 Pro+ cell line expressed high amounts of P5CS. To ensure similar total protein levels between the different cell lines, GAPDH expression was analyzed. As shown in Figure 10, total protein levels showed only slight variations between the different CHO cell lines. In addition, OAT expression levels were analyzed. As a positive control, HEK cells giving a strong OAT and P5CS signal at lower total protein levels were used. None of the analyzed CHO cell lines, neither parental proline-auxotroph (Pro-), nor proline-prototroph (Pro+) cell lines, showed a detectable OAT expression.

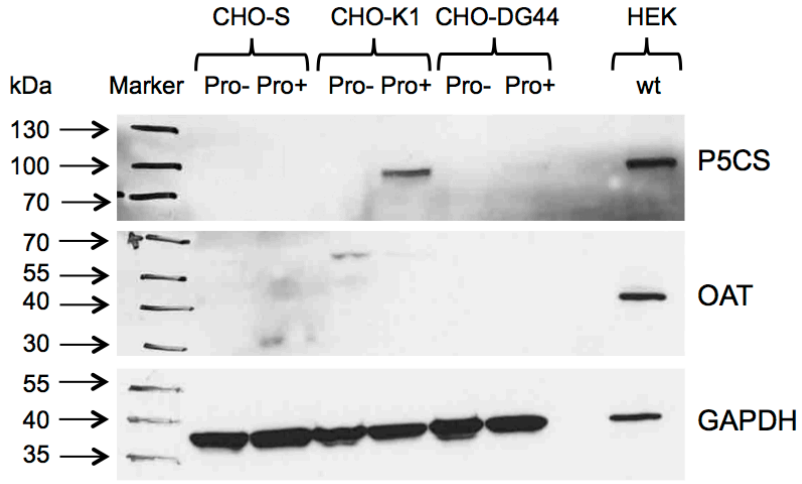

Figure 10: Western Blot analysis of P5CS and OAT in different Pro- and Pro+ CHO cell lines
10^7 Pro- or Pro+ CHO-K1, CHO-S or CHO DG44 and HEK cells were lysed in 1 mL RIPA buffer and after three 10 sec 30% sonification pulses, 70 μg protein (only 20 μg for HEK cells) per lane were separated by SDS-PAGE (4-20% Tris-Glycine gel). Proteins were transferred onto a nitrocellulose membrane and stained with anti-P5CS or anti-OAT primary antibodies and anti-rabbit HRP coupled secondary antibody or directly with an anti-GAPDH HRP-coupled antibody. The membranes were developed using an HRP substrate.

3.1.1.3 Genetic background of the Pro+ phenotype

To analyze the genetic background of the reversion from the Pro- to the Pro+ phenotype, the cDNA sequence and mRNA levels of P5CS and OAT were analyzed. Since the hamster genome sequence was not available at that point in time the P5CS and OAT ORF of ten different species (Table 1) were aligned (Appendix 1, Appendix 2). These alignments were used to design several normal and degenerated primers. RNA was prepared from Pro+ and Pro- CHO-K1 cell lines and translated into cDNA. The cDNAs were amplified using the designed P5CS- and OAT-specific primers in a PCR and were then sequenced directly as a PCR product or after cloning into pGEM-T Easy vectors to facilitate better sequencing efficiencies. Sequence analysis of Pro+ compared to the Pro- P5CS ORF from CHO-K1 cells revealed no difference in the nucleotide sequence (Appendix 3).

Table 1: Organisms used for sequence alignment to generate P5CS and OAT specific primer

Dipodomys ordii	Kangaroo rat
Oryctolagus cuniculus	Rabbit
Mus musculus	Mouse
Rattus norvegicus	Rat
Canis familiaris	Dog
Bos taurus	Cattle
Equus caballus	Horse
Spermophilus tridecemlineatus	Thirteen-lined ground squirrel
Cavia porcellus	Guinea pig
Homo sapiens	Human

In addition, the mRNA levels of P5CS and OAT in CHO-S and CHO-K1 Pro+ cells were compared with respect to Pro- cells using real-time PCR. The target genes (OAT and P5CS) of CHO-K1 Pro- and the reference gene (GAPDH) were amplified via PCR from cDNA and cloned into the pGEM-T Easy vector as described above. Real-time PCR using dilutions of these control vectors indicated that all three genes had similar efficiencies allowing a ΔΔ-CT analysis. The mRNA of CHO-K1 and CHO-S Pro- and Pro+ was isolated, transcribed into cDNA and analyzed comparing P5CS or OAT expression against the reference gene GAPDH. As shown in Figure 11, P5CS mRNA levels were increased in both Pro+ CHO cell lines when compared to the Pro- cell lines. However, the up-regulation did not directly correlate with protein levels. In the CHO-S Pro+ cell line, the P5CS mRNA level was about 21.9-fold increased and about 4.5-fold in the CHO-K1 Pro+ cell line. In contrast, no significant increase of OAT mRNA levels could be observed in the Pro+ cell lines. The fold change of Pro+ compared to Pro- ranged from 1.7 to 1.9.

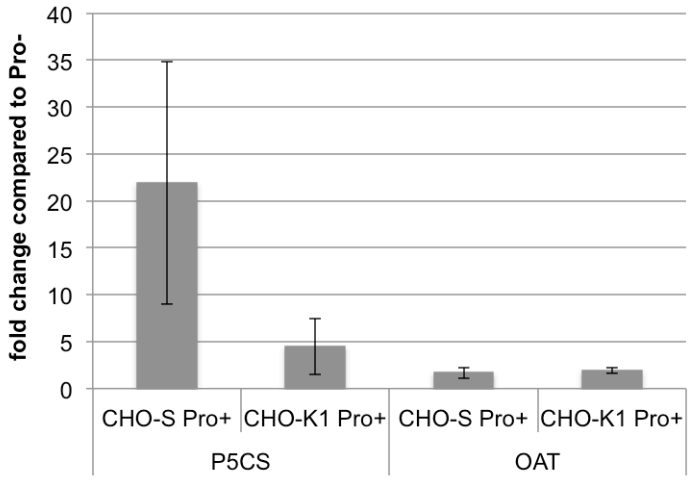

Figure 11: ΔΔ-CT analysis of real-time PCR data detecting P5CS and OAT in Pro+ compared to Pro- CHO-S and CHO-K1 cells

P5CS and OAT cDNA levels were detected in a semi-quantitative real-time PCR and were normalized by GAPDH expression levels. For ΔΔ-CT analysis the fold change of CHO-S and CHO-K1 Pro+ cell lines compared to Pro- parental cell lines was calculated (n= 3-4).

It can be concluded that the proline prototroph phenotype was caused by an increased expression of P5CS and that this up-regulation was dependent on the used CHO cell line.

3.1.2 Generation of the dicistronic pMACS-CHO expression vector

The pMACS 4-IRES.II vector (Figure 12 A) was used as a backbone to generate the pMACS-CHO expression vector (Figure 12 B) with coupled expression of GOI and selection marker. This backbone comprised a bacterial amplification site, consisting of an ampicillin resistance gene and an origin of replication, a CMV5 promoter (human CMV immediate early promoter and enhancer fused with the tripartite leader and splicing site from the human adenovirus 5 major late promoter) and an EMCV IRES. The multiple cloning site (MCS) and the intervening sequence (IVS) between promoter and IRES were replaced by a small synthetic intron (donor site from first intron of human beta-globin gene and branch/acceptor site from intron of an immunoglobulin gene) and a new MCS featuring different restriction enzyme sites. The CD4 cDNA and PolyA behind the IRES were replaced by a second MCS and a BGH PolyA sequence.

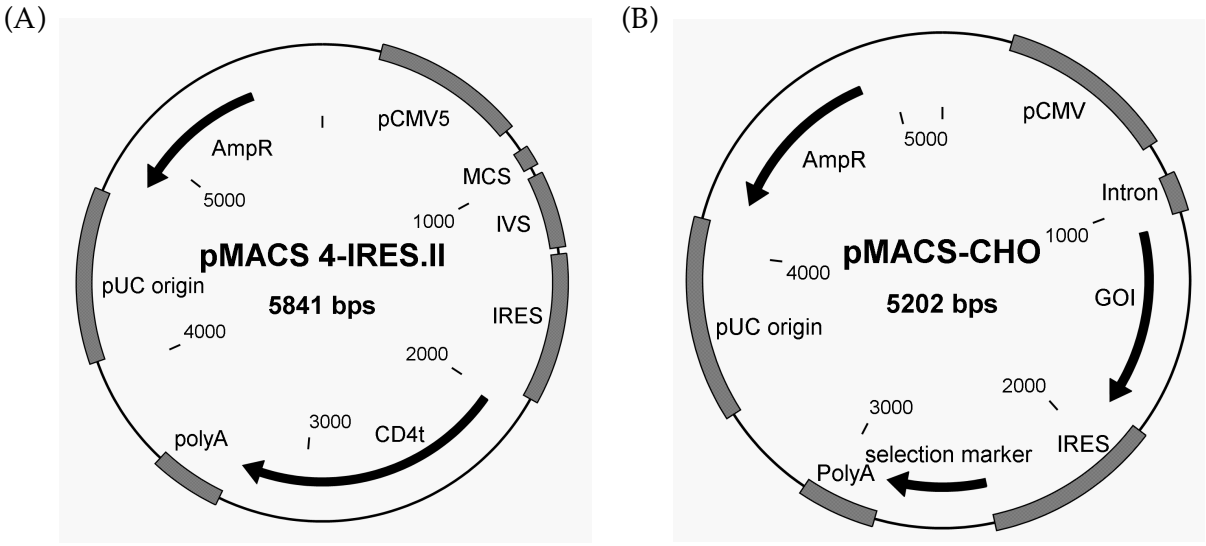

Figure 12: Vector maps of the pMACS 4-IRES.II used for MACS-isolation of transiently transfected cells and the new designed and generated pMACS-CHO expression vector

The bacterial amplification site, promoter and IRES were used from the original pMACS 4-IRES.II vector (A). Integration of new multiple cloning sites, a synthetic intron, a BGH PolyA signal and different GOIs and selection markers generated the pMACS-CHO expression vector (B).

The GOIs were integrated into this novel pMACS-CHO expression vector in the first MCS in front of the IRES. The latter were cDNA sequences of different model proteins: GFP, heavy and light chain of a humanized anti-CD303 antibody (humanized variable regions, human IgG1 and kappa constant regions), heavy and light chain of chimeric anti-Biotin and anti-CD14 antibodies (murine variable regions, human IgG1 and kappa constant regions) and human TGF-β1 with a signal sequence of α-Fibrinogen (FGA). Selection markers, again

integrated as cDNA sequences into the second multiple cloning site behind the IRES, included: mouse P5CS (Aldh18a1) transcription variant 1, neomycin, hygromycin B and zeocin resistance genes and CHO-K1 GS. All sequences were either synthetically produced (DNA2.0) and codon optimized for expression in mammalian cells or cloned from different commercial vectors. All gene sequences comprised the Kozak sequence "GCC GCC ACC" in front of the start codon for optimized initiation of translation.

3.1.2.1 Characterization of pMACS-CHO in transient transfections

To determine the characteristics of the pMACS-CHO expression vector, several different constructs (pAS-5, pAS-8, pAS-16, pAS-17, pAS-18) were transiently transfected into CHO-K1 cells and GFP expression was monitored by flow cytometry 48 hours after transfection. To determine whether the second gene behind the IRES does influence the first gene in front of the IRES in transient transfections, the vectors contained GFP as a GOI and either no gene or different selection marker genes behind the IRES. As shown in Table 2, the expression intensity measured as GFP mean fluorescence intensity (MFI) was reduced from 9% to 31% due to the second gene in five independent transfections at similar transfection efficiencies.

Table 2: Influence of the second gene on the expression of the first gene in the pMACS-CHO vector in transient transfections

CHO-K1 cells were transfected with the vectors pAS-5, pAS-8, pAS-16, pAS-17 or pAS-18 and the GFP fluorescence was analyzed by flow cytometry 48 hours after transfection (n=5).

Vector	Vector description	Size of gene behind IRES	% GFP		GFP MFI	
			Mean	Standard deviation	Mean	Standard deviation
pAS-5	GFP-IRES	0 bp	46.7	6.7	567.4	68.5
pAS-8	GFP-IRES-neoR	1041 bp	40.8	4.7	511.6	73.3
pAS-16	GFP-IRES-P5CS	2439 bp	38.7	8.4	408.5	63.9
pAS-17	GFP-IRES-GS	1176 bp	44.3	3.6	478.0	47.7
pAS-18	GFP-IRES-hygroR	1062 bp	37.2	5.6	391.1	60.2

In parallel, the vectors pAS-5, pAS-9, pAS-31, pAS-32 were transiently transfected and the GFP fluorescence was monitored by flow cytometry after 48 hours. This set of experiments was dedicated to find out how strong the expression of the second gene is compared to the first gene and whether the integration of a first gene influences the IRES-dependent translation. As shown in Table 3, the expression intensity of GFP at the second position behind the IRES was only 3% of the CAP-translated intensity at the first position. Integration of two different sequences in front of the IRES did not further reduce the expression of the IRES-dependent GFP. In fact, an even higher GFP MFI could be observed with one of the sequences (pAS-32).

Table 3: Influence of the second gene on the expression of the first gene in the pMACS-CHO vector in transient transfections

CHO-K1 cells were transfected with the vectors pAS-5, pAS-9, pAS-31 or pAS-32 and the GFP fluorescence was analyzed by flow cytometry 48 hours after transfection (n=5).

Vector	Vector description	Size of gene before IRES	% GFP		GFP MFI	
			Mean	Standard deviation	Mean	Standard deviation
pAS-5	GFP-IRES	-	46.7	6.7	567.4	68.5
pAS-9	IRES-GFP	0 bp	18.1	6.2	18.4	2.0
pAS-31	HC anti-CD-303-IRES-GFP	1098 bp	7.4	1.8	16.0	1.2
pAS-32	LC anti-CD303-IRES-GFP	723 bp	18.7	4.0	34.6	3.7

3.1.2.2 Comparison of different vector designs

The newly generated pMACS-CHO expression vector (Figure 12 B) features a coupled expression of GOI and selection marker, due to an IRES site. It was compared to a pSV2-hCMV (pSV2) vector with a two-promoter strategy. Both vectors comprise the same hCMV promoter, GFP as GOI and neomycin resistance gene as selection marker. In the pSV2-hCMV vector, the expression of the selection marker is under control of a SV40 early promoter. Both vectors (pSV2neo-hCMV-GFP and pAS-8) were transfected (T4.3 and T4.4) into CHO-K1 cells in two separate transfections. Stable cell lines were generated by neomycin selection and GFP expression was monitored at every passage using flow cytometry. For or all used antibiotic selection markers, optimal antibiotic concentrations were determined by titration in killing curves with parental cells and by testing of different concentrations during the selection process (data not shown). As shown in Figure 13 A, stable cell lines generated with the pMACS-CHO vector (pAS-8) showed high GFP producer rates of 78% to 90% on day 20 after transfection, while still increasing over culture time and reaching up to 99% GFP producers around day 40 after transfection. Stable cell lines generated using the pSV2 vector (pSV2neo-hCMV-GFP) only reached maximum GFP producer rates of 32% declining over culture time. In addition, differences can also be seen in the MFI of the GFP signal. The MFI of cell lines generated by transfection of the pMACS-CHO vector ranged from 70 to 150. Cell lines generated by transfection of the pSV2 vector had a much lower MFI, ranging from 20 to 40 (Figure 13 B).

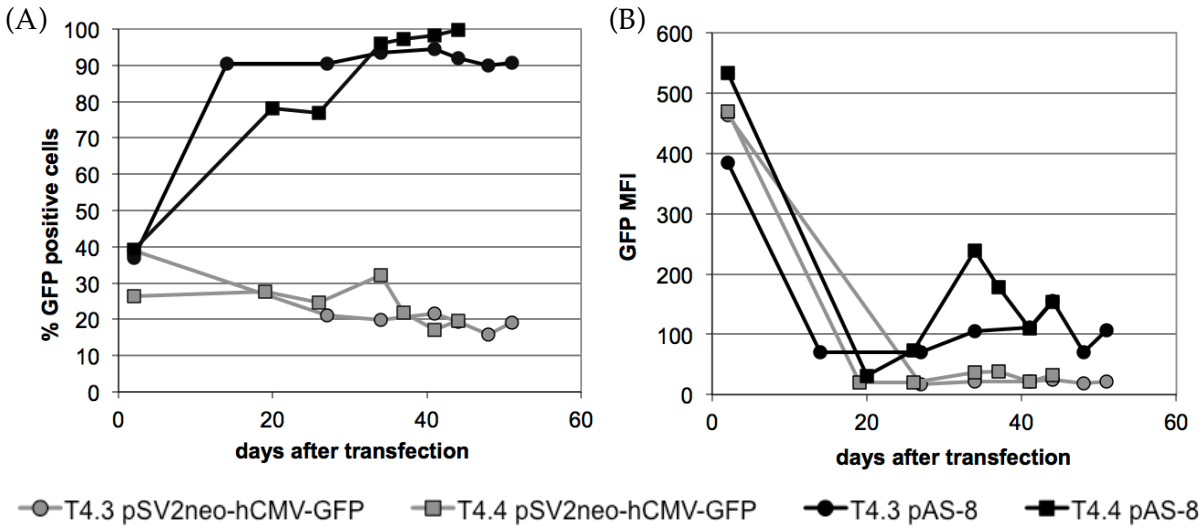

Figure 13: Stable cell lines expressing GFP generated by different vector designs
Expression vectors with separate expression cassettes of GFP and neomycin selection marker (pSV2) or IRES coupled expression (pMACS-CHO) were compared in a stable cell line generation process (T4.3/4.3 pSV2-hCMV-GFP or pAS-8): (A) number of GFP positive cells, (B) GFP MFI.

Thus, although integration of an IRES-dependent gene into the pMACS-CHO vector did reduce the expression levels of the CAP-dependent gene in transient transfection, the coupling of GOI and selection marker led to stable cell lines with high producer rates and expression levels.

3.1.3 P5CS as a novel selection marker

For first studies of the suitability of P5CS as a selection marker in CHO cells, the enhanced GFP was chosen as GOI, due to its ease of detection in flow cytometry and microscopy. CHO-K1 cells were transfected (T4.4) with the vector pAS-16 (pMACS-CHO, GOI: GFP, selection marker: P5CS) and stable cell lines were generated in a proline-free selection medium containing 10% dialyzed FBS (MEM supplemented with glutamine and 10% dFBS). At each passage the stable cell lines were analyzed for their GFP fluorescence by flow cytometry. Using P5CS as selection marker, it was possible to generate stable cell lines (T4.4 pAS-16 I and II) with up to 98% GFP-expressing cells and stable GFP MFI (Figure 14). As one drawback the long selection time must be mentioned. Up to 100 days were required to reach producer rates of over 90%. In about 50% of all generated cell lines, it was possible to generate high producer cell lines with over 90% GFP-positive cells (e.g. T4.4 pAS-16 II). In other cases the producer rate stagnated or even decreased again (e.g. T4.4 pAS-16 I).

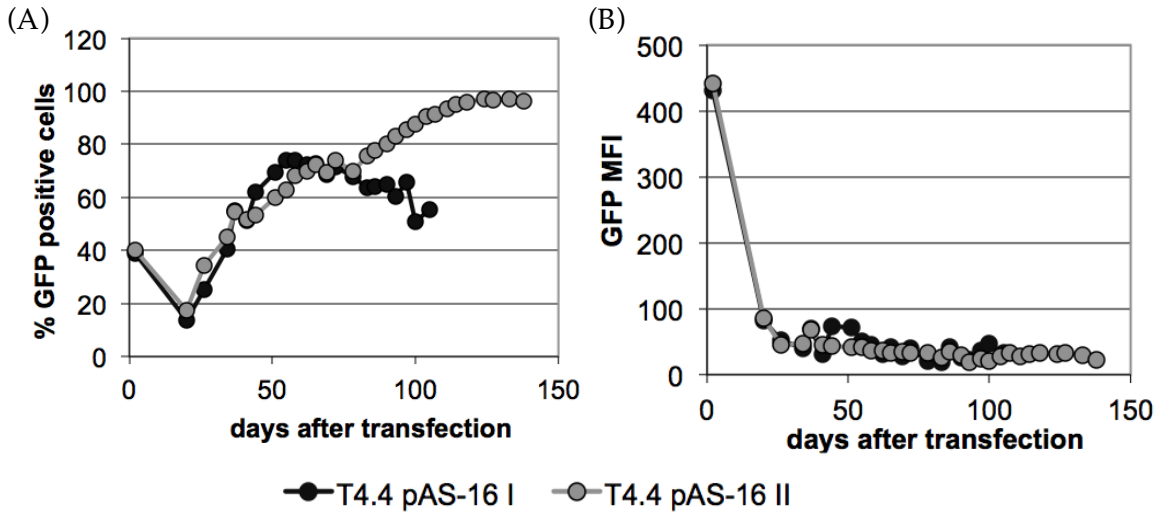

Figure 14: Stable GFP-expressing CHO-K1 cell lines selected by P5CS in proline-free medium

CHO-K1 cells were transfected (T4.4) with the vector pAS-16 and selected in MEM supplemented with 10% dialyzed FBS. GFP expression was monitored at every passage using flow cytometry: (A) Percentage of GFP positive cell, (B) GFP MFI.

Since Western Blot analysis of P5CS protein levels of Pro+ cells and parental Pro- cell lines revealed differences between CHO cell lines (Figure 10), it was suspected that the different CHO cells might also differ in their sensitivity to P5CS selection. Therefore, the stable cell line generation process was tested with the different CHO cell lines CHO-K1, CHO-S and CHO DG44 (T4.4/4.11 pAS-16) and the rate of GFP-positive cells was again monitored. As shown in Figure 15, the selection times of different CHO cell lines showed large variations. On day 50 after transfection, a stable CHO-S cell line (CHO-S T4.11 pAS-16) had a GFP-producer rate of 79% compared to a CHO-K1 cell line (CHO-K1 T4.4 pAS-16) with 60% and a CHO DG44 cell line (CHO DG44 T4.11 pAS-16) with only 27% GFP-producers. Similarly, the GFP MFI was highest in the CHO-S cell line, followed by CHO-K1 and CHO DG44 cell lines.

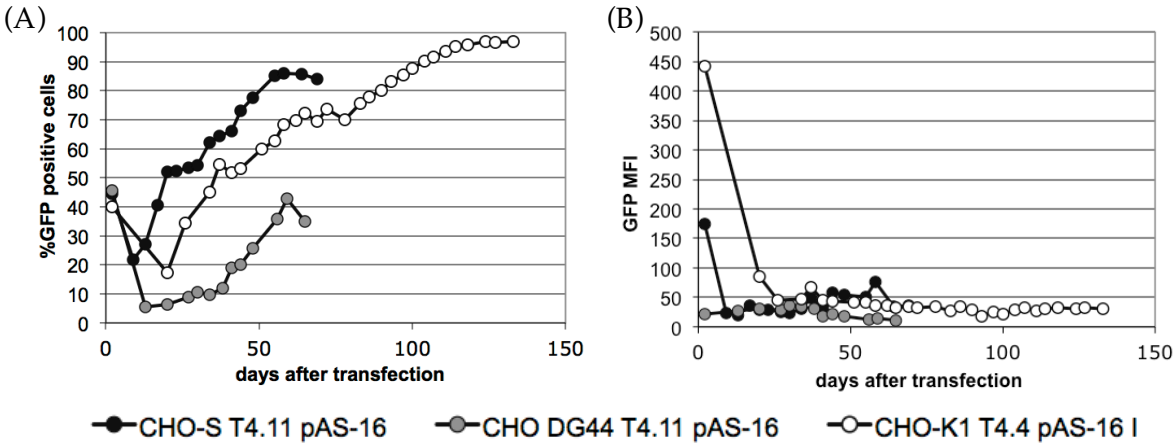

Figure 15: Test of feasibility of different CHO cell lines in a P5CS selection system

CHO-K1, CHO-S and CHO DG44 cells were transfected (T4.4/4.11) with the vector pAS-16 and selected in MEM supplemented with 10% dialyzed FBS. GFP expression was monitored at every passage using flow cytometry: (A) Percentage of GFP positive cell, (B) GFP MFI.

To further improve selection times, MEM supplemented with glutamine, ITS+3 and a reduced dialyzed FBS concentration of 5% was tested in the cell line generation process. The reduction of dialyzed FBS led to a reduction of proline traces in the medium. Amino acid analysis of the used FBS lot revealed a proline concentration of 0.39 mM proline (Appendix 4). Using 10% FBS supplement in the medium, this resulted in a concentration of 0.039 mM proline, while using 5% FBS resulted in 0.0195 mM. Reduction of proline concentration dramatically improved the selection time in all tested CHO cell lines. In CHO-K1 cells it was possible to reach over 90% GFP-positive cells within 16 days (Figure 16, T4.44 pAS-16 I) instead of over 100 days (Figure 16, T4.17 pAS-16), previously.

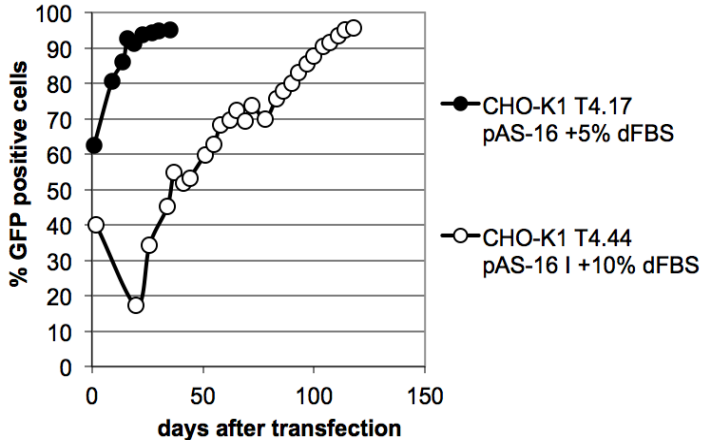

Figure 16: Influence of FBS concentrations in selection medium on P5CS selection system
CHO-K1 cells were transfected (T4.17) with the vector pAS-16 and selected in MEM supplemented with ITS+3 and with 5% or 10% dialyzed FBS. Number of GFP positive cells was monitored at every passage using flow cytometry.

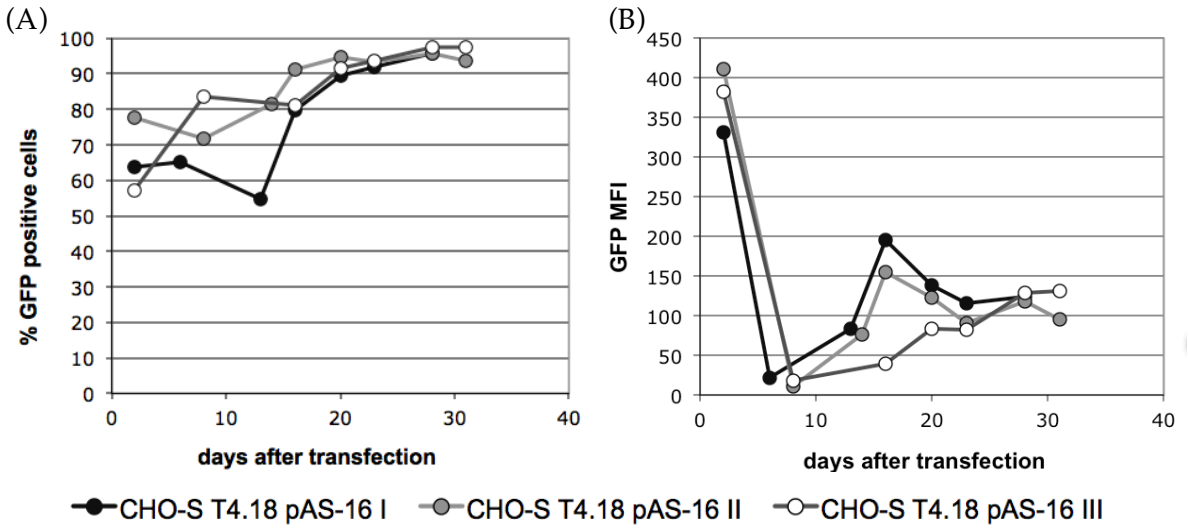

Figure 17: Reproducible and fast P5CS selection in CHO-S cells
CHO-S cells were transfected (T4.18) with the vector pAS-16 and selected in CHO-MEM supplemented with 5% dialyzed FBS. GFP expression was monitored at every passage using flow cytometry: (A) Percentage of GFP positive cell, (B) GFP MFI.

Transfecting the CHO-S cell line in triplicates and generating stable cell line (T4.18 pAS-16 I, II and II) in the serum-reduced selection medium with 5% dialyzed FBS showed that reproducible producer rates of over 90% in 20 days with constant GFP MFIs could be reached with the novel P5CS selection (Figure 17 A and B).

From these experiments it was concluded that the CHO-S cell line might be the most suitable one for the P5CS selection system. Using CHO-S cells allowed fast and reproducible stable cell line generation for the expression of a fluorescent protein.

3.1.3.1 P5CS as a selection marker for the expression of monoclonal antibodies

To test the performance of the P5CS selection system for the expression of monoclonal antibodies, a two-vector strategy was chosen. In a first transfection (T4.14 pAS-21+23) the anti-CD303 antibody was expressed in CHO-S cells and stable cells were selected by a combination of P5CS selection for the heavy chain and neomycin selection for the light chain in a selection medium containing 10% dialyzed FBS. The isolated stable cell line produced about 100 ng/mL antibody. Clones were isolated by manual clone picking from a semi-solid medium containing a FITC-labeled antibody to stain secreted antibody. The isolated clones produced between 95 and 364 ng/mL (Figure 18).

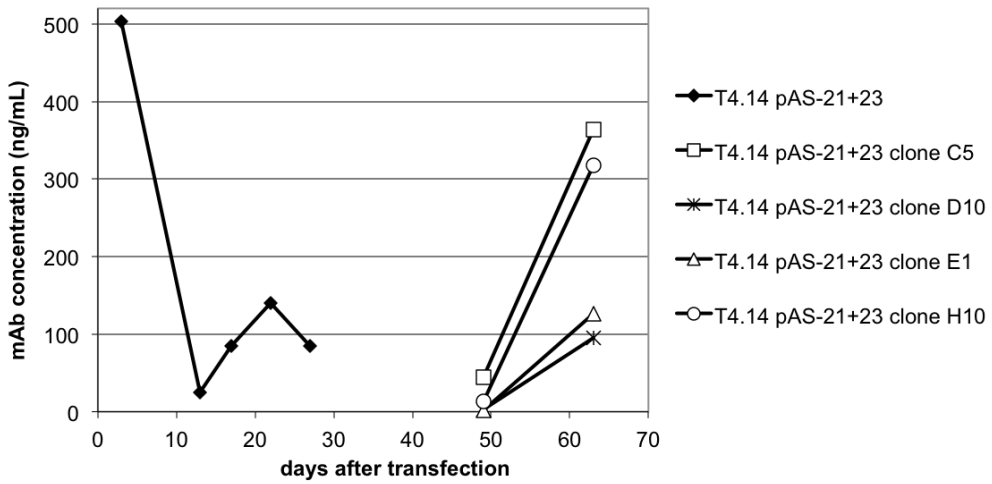

Figure 18: Stable cell line expressing anti-CD303 and isolated clones thereof

CHO-S cells were cotransfected with pMACS-CHO vectors (T4.14 pAS-21 and pAS-23) encoding for heavy and light chain of the anti-CD303 antibody. Stable cells were selected by P5CS and neomycin selection. Clones were isolated from the stable cell line by a manual clone picking process from semi-solid medium with identification of producing clones by labeling of secreted antibody via a FITC-conjugated detection antibody.

Similar to the expression of GFP as a model protein, the reduction of dialyzed FBS from 10% to 5% greatly enhanced the productivity of isolated cell lines. Furthermore, different selection protocols such as different splitting rates 24 hours after transfection and different points in time of addition of the selection medium (24 hours, 48 hours or 72 hours after

transfection) were tested (data not shown). Experiments indicated that a harsh split (1:20) together with the addition of selection medium (both removal of proline and addition of antibiotics) 24 hours after transfection resulted in the highest titers. As a low serum proline-free medium the CHO-MEM medium was developed containing 5% dialyzed FBS and various supplements to enhance growth and productivity (data not shown). As mentioned above, similar results were obtained expressing GFP in a one-vector strategy using the P5CS selection system only. To further improve the selection system, expression vectors containing heavy or light chain genes were combined with different selection markers. Stable cell lines expressing anti-CD303 were generated using the low serum (5%) protocol and productivity was measured as maximum antibody concentrations in the supernatant of overgrown cultures (T4.20/25 pAS-21+23, pAS-19+25, pAS-21+55, pAS-25+54, pAS-21+47, pAS-25+46). Of all tested selection marker combinations the expression of the heavy chain using P5CS selection and expression of the light chain using zeocin selection resulted in the highest antibody titers with a mean of 3.7 μg/mL (Figure 19).

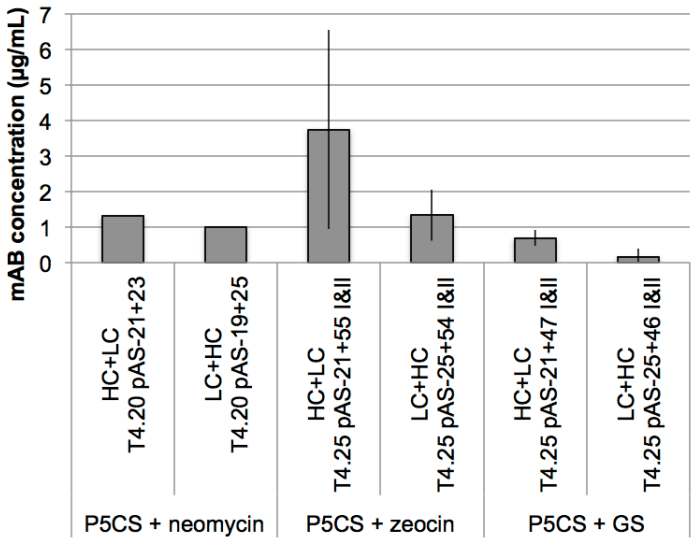

Figure 19: **Comparison of different selection marker combinations for generation of anti-CD303-expressing cell lines**

CHO-S cells were cotransfected (T4.20/25) with pMACS-CHO vectors encoding for heavy and light chain of the anti-CD303 antibody containing different selection markers (pAS-21+23, pAS-19+25, pAS-21+55, pAS-25+54, pAS-21+47, pAS-25+46). Cells were selected in CHO-MEM supplemented with 5% dialyzed FBS missing corresponding amino acids (P5CS: proline, GS: glutamine) or containing antibiotics needed for selection. An IgG/kappa-specific ELISA was used to monitor the antibody titers in supernatants of overgrown cultures on day 55 after transfection (n=1-2).

In further experiments, the combination of HC-P5CS and LC-zeocin selection was compared to a selection using antibiotics only. A neomycin resistance gene for the heavy chain selection replaced the P5CS, while the zeocin resistance gene remained on the vector for the light chain selection. The generated cell lines (T4.25 pAS-21+55 I and II or pAS-19+55 I and II) produced about 1-2 mg/L monoclonal antibody on day 45 after transfection using a

combination of P5CS and zeocin selection or a combination of neomycin and zeocin selection (Figure 20). At around day 60, the stable producer cell lines showed an additional increase in titers. Using P5CS combined with zeocin selection antibody titers of up to 8 mg/L were reached in minimal medium (CHO-MEM) and up to 4 mg/L using neomycin and zeocin selection.

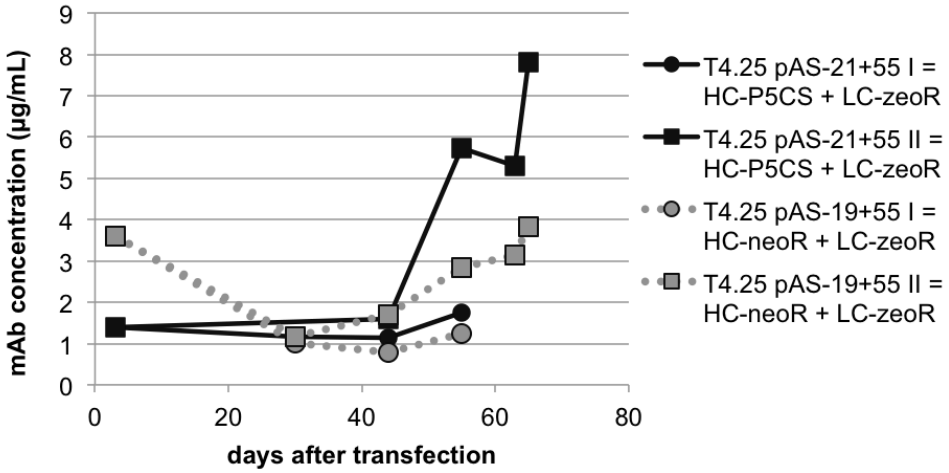

Figure 20: Comparison of P5CS/zeocin selection to neomycin/zeocin selection for generation of anti-CD303-expressing cell lines

CHO-S cells were cotransfected (T4.25) with pMACS-CHO vectors (pAS-19+55 or pAS-21+55) encoding for heavy and light chain of the anti-CD303 antibody containing different selection markers. Cells were selected by proline-free zeocin-containing CHO-MEM or by a proline-supplemented zeocin- and neomycin-containing CHO-MEM supplemented with 5% dialyzed FBS. The antibody titers in supernatants of overgrown cultures were monitored by an IgG/kappa-specific ELISA.

Intracellular staining of the cell lines T4.25 pAS-19+55 II (= HC-neomycin+LC-zeocin) and pAS-21+55 II (= HC-P5CS+LC-zeocin) on day 72 after transfection using an APC-labeled anti-human IgG antibody and a FITC-labeled anti-human kappa antibody analyzed by flow cytometry (Figure 21) revealed double producer rates of heavy and light chain of 85% for the P5CS/zeocin selection (Figure 20: T4.25 pAS-21+55 II) and 77% for the neomycin/zeocin selection (Figure 20: T4.25 pAS-19+55 II).

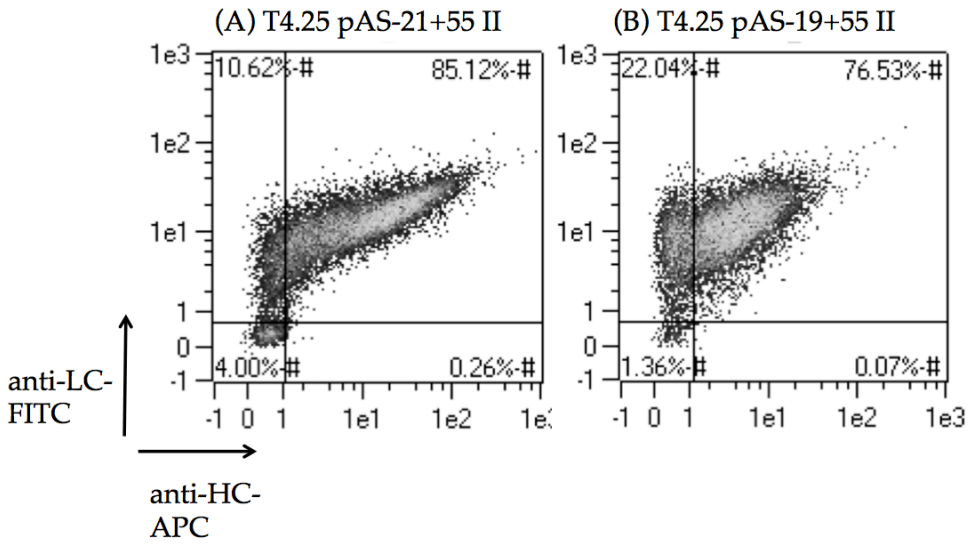

Figure 21: Intracellular staining of IgG (HC) and kappa (LC) in stable anti-CD303-expressing CHO-S cell lines
CHO-S cells expressing anti-CD303 selected by (A) P5CS/zeocin (T4.25 pAS-21+55 II) or (B) neomycin/zeocin (T4.25 pAS-19+55 II) selection were fixed and permeabilized. Intracellular IgG chains were stained with a specific antibody coupled to APC and intracellular kappa chains were stained with a specific antibody coupled to FITC. APC and FITC signals were monitored by flow cytometry.

On day 57 after transfection, a limiting dilution of the highest producing cell lines (Figure 20: T4.25 pAS-21+55 II and pAS-19+55 II) for each selection marker combination was performed. After about 20 days, the supernatants of 75 to 81 wells containing single clones were screened in an ELISA. The determined antibody concentrations are shown in Figure 22. The titer distribution of clones isolated by limiting dilution confirmed the high producer rates determined by previous intracellular staining of the starting producer cell lines, as hardly any of the screened clones secreted no antibody.

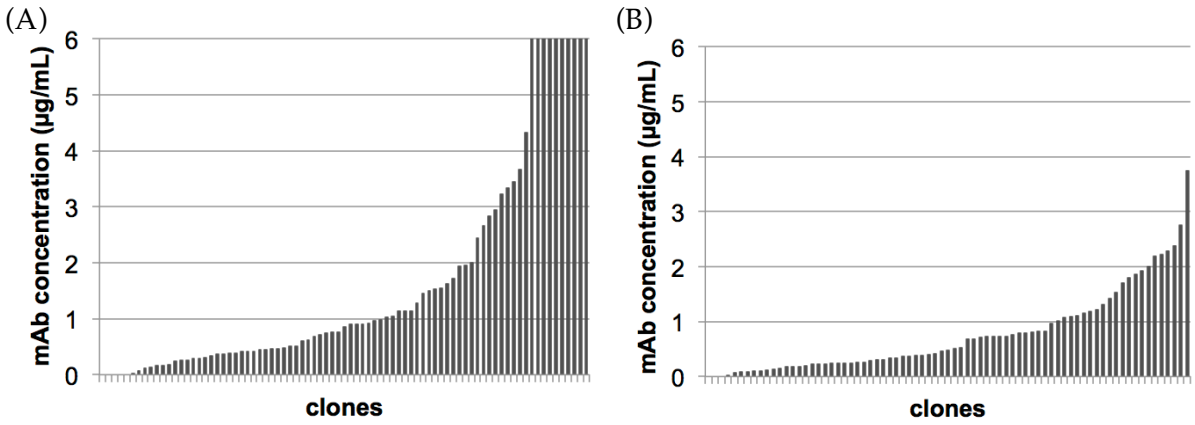

Figure 22: Clone distribution of clones isolated by limiting dilution from the stable cell lines T4.25 pAS-21+55 II or pAS-19+55 II
Stable CHO-S cell lines expressing anti-CD303 selected by (A) P5CS/zeocin (T4.25 pAS-21+55 II) or (B) neomycin/zeocin (T4.25 pAS-19+55 II) were subjected to a limiting dilution. Supernatants of 96-wells containing a clone were screened in IgG/kappa-specific ELISA.

All clones expressing more than 0.5 mg/L in the first screen were expanded. For further analysis $2x10^5$ cells were seeded per 12-well. After 52 hours, cells were counted to determine the clones´ growth properties. Also, the antibody concentration of the supernatants was determined at that point in time (Figure 23). For both selection marker combinations proliferation during the 52 hours ranged from no expansion to doubling times of 26 hours (data not shown). However, the range of observed proliferation rates is comparable between the different selection marker combinations. The amount of antibody produced by $1x10^5$ cells during the culture period ranged from 0.4 to 1.71 μg/mL.

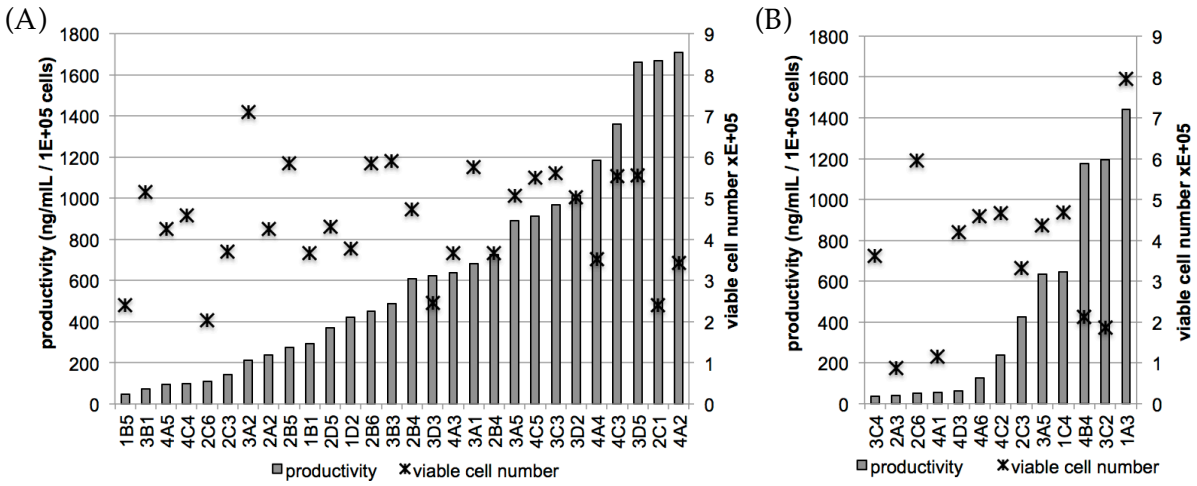

Figure 23: Clone analysis of clones isolated by limiting dilution from the stable cell lines T4.25 pAS-21+55 II or pAS-19+55 II

CHO-S clones expressing anti-CD303 were isolated by limiting dilution from (A) T4.25 pAS-21+55 II or (B) T4.25 pAS-19+55 II. Best producing clones of the 96-well screen were expanded. For analysis $2x10^5$ cells of these clones were seeded per 12-well and antibody titer as well as viable cell number were determined after 52 hours. The produced antibody amount during this time frame was calculated per $1x10^5$ cells.

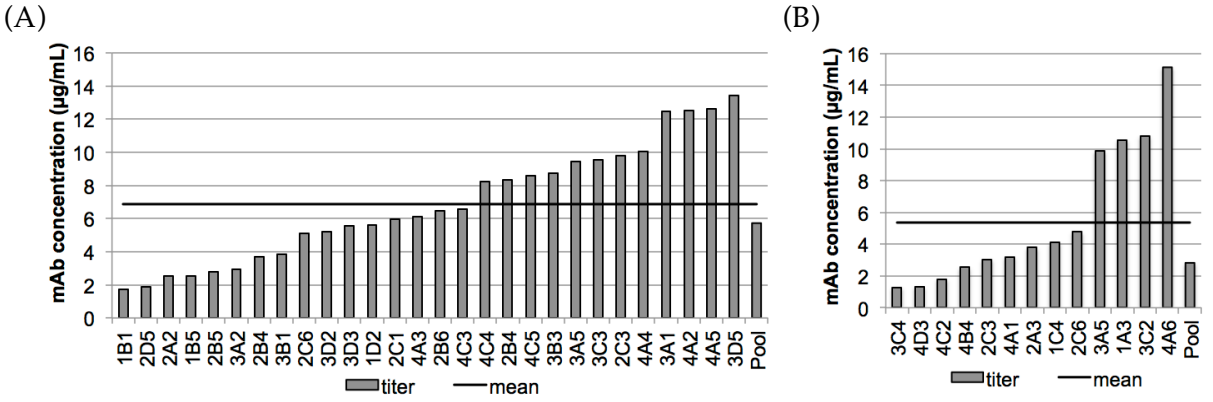

Figure 24: Clone analysis of clones isolated by limiting dilution from the stable cell lines T4.25 pAS-21+55 II or pAS-19+55 II

CHO-S clones expressing anti-CD303 were selected by (A) P5CS/zeocin (T4.25 pAS-21+55 II) or (B) neomycin/zeocin (T4.25 pAS-19+55 II). Best producing clones of the 96-well screen were expanded. For analysis $2x10^5$ cells of these clones were seeded per 12-well and the antibody titer of overgrown cultures was determined by an IgG/kappa-specific ELISA.

As an additional parameter for clone selection, a second 12-well with $2x10^5$ seeded cells was used to determine the maximum titer (Figure 24). For the analyzed clones selected by P5CS and zeocin, the mean titer amounted to 6.9 $\mu g/mL$, with individual clones ranging from 1.7 to 13.4 $\mu g/mL$. In comparison, the mean of all analyzed clones isolated with neomycin and zeocin selection was only 5.4 $\mu g/mL$, with individual clones ranging from 1.2 to 15.1 $\mu g/mL$. The mean titer of clones selected by P5CS and zeocin was 1.3-fold higher than that of clones selected by neomycin and zeocin.

To verify the usability of the P5CS selection system for the expression of other monoclonal antibodies, the cell line generation process and the clone isolation by limiting dilution were repeated for two additional antibodies, anti-Biotin (T4.32 pAS-97+100 II or pAS-98+100 I and II) and anti-CD14 (T4.33 pAS-102+105 I and II or T4.32 pAS-103+105 I and II). Both antibodies are encoded by mouse variable regions and human constant regions (IgG1 and kappa). For both test constructs, it was possible to generate stable producer cell lines with titers of about 0.2-1.5 $\mu g/mL$, as shown in Figure 25.

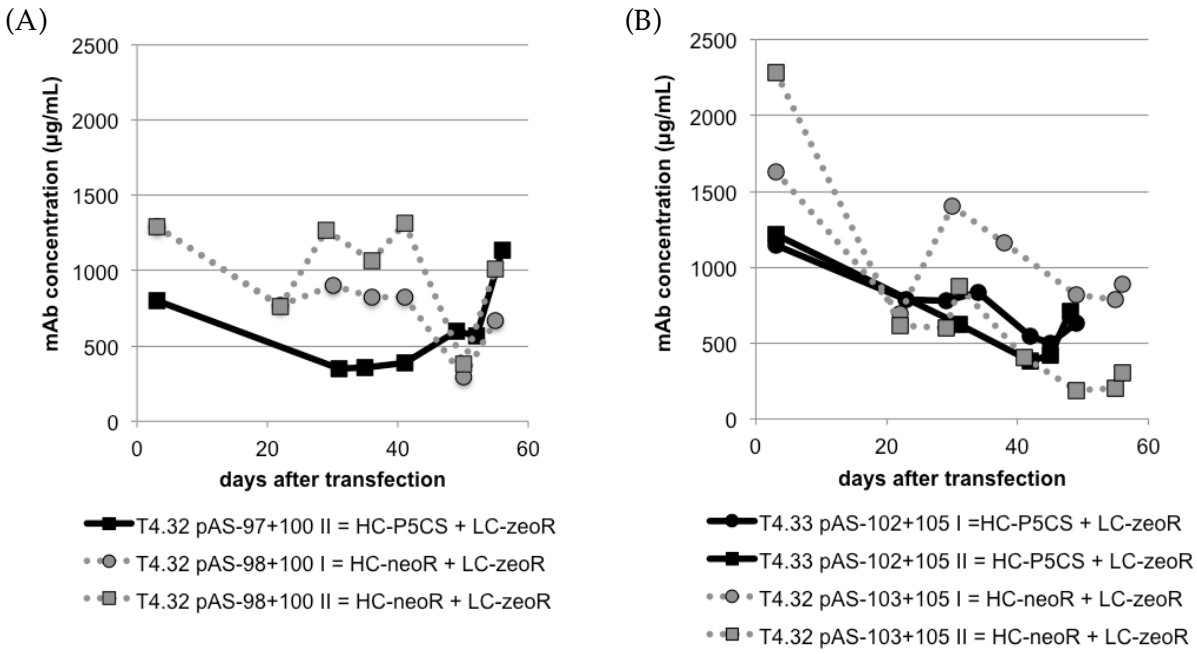

Figure 25: **Comparison of P5CS/zeocin selection to neomycin/zeocin selection for generation of anti-Biotin- and anti-CD14-expressing cell lines**

CHO-S cells were cotransfected (T4.32/4.33) with pMACS-CHO vectors for heavy and light chain of the (A) anti-Biotin (pAS-97+100 or pAS-98+100) or (B) anti-CD14 (pAS-102+105 or pAS-103+105) antibody containing different selection markers. Cells were selected in a proline-free, zeocin-containing or a proline-supplemented, zeocin- and neomycin-containing CHO-MEM supplemented with 5% dialyzed FBS. The antibody titers in supernatants of overgrown cultures were monitored by an IgG/kappa-specific ELISA over time.

Analysis of the titer distribution of clones isolated by limiting dilution from the stable producer cell lines (T4.32 pAS-97+100 II, T4.32 pAS-98+100 II, T4.33 pAS-102+105 I or T4.32 pAS-103+105 I) on day 23 to 37 after transfection again revealed high producer rates of 93%

to 98% (Table 4). Maximum titers of clones isolated by limiting dilution ranged from 3.9 μg/mL for anti-Biotin to 3.2 μg/mL for anti-CD14. Mean titers ranged from 1.0 μg/mL for anti-Biotin to 0.8 μg/mL for anti-CD14. In contrast to the model antibody anti-CD303, for both anti-CD14 and anti-Biotin the titers of clones isolated by P5CS/zeocin selection were 1.4 to 2.0-fold lower compared to neomycin/zeocin selection.

Table 4: **Comparison of P5CS/zeocin and neomycin/zeocin selection for the expression of three monoclonal antibodies in CHO-S stable cell lines or clones isolated by limiting dilution.**
Stable cell lines expressing anti-CD303 (T4.25 pAS-21+55 II or pAS-19+55 II), anti-Biotin (T4.32 pAS-97+100 II or pAS-98+100 II) or anti-CD14 (T4.33 pAS-102+105 I or T4.32 pAS-103+105 I) were used to isolate clones by limiting dilution. Upgrowing clones were screened in the 96-well plate for antibody titers. The producer rates were calculated from the number of clones expressing antibody and the total amount of screened clones.

		Starting cell line titer (μg/mL)	Number of screened clones	Maximal titer clones (μg/mL)	Mean titer clones (μg/mL)	Producer rate
anti-CD303	P5CS +zeocin	5.73	81	>6.50	1.68	91%
	neomycin +zeocin	2.83	75	3.75	0.76	97%
anti-Biotin	P5CS +zeocin	0.36	53	1.76	0.55	98%
	neomycin +zeocin	1.07	83	3.90	1.04	94%
anti-CD14	P5CS +zeocin	0.83	124	2.14	0.58	98%
	neomycin +zeocin	1.17	67	3.17	0.81	91%

Based on the results obtained by expressing different recombinant antibodies, it was concluded that the developed P5CS system might lead to similar expression levels as antibiotic selections.

3.1.3.1.1 Batch production in suspension shake cultures

The ten highest producing clones (of the stable cell line T4.25 pAS-21+55 II) expressing the anti-CD303 antibody isolated by P5CS/zeocin selection and limiting dilution (Figure 20 and Figure 24) were adapted to the serum-free and zeocin-containing production medium CHO MACS CD custom-made without proline. The cells were incubated in Erlenmeyer flasks in an orbital shake reactor. After a period of about four weeks to allow adaptation to serum-free conditions and the shake incubator, viability recovered to over 90% and clones were subjected to a determination of specific productivities. Therefore, clones were seeded at $2x10^5$ cells/mL and each day an aliquot was taken to measure antibody titer and cell number (Figure 26 and Table 5). For all analyzed clones, an increase in maximum titers of 2- to 6-fold compared to static, adherent culture condition could be observed. The highest antibody concentration was produceded by clone 4A2 and amounted to 63 mg/L. Viable cell densities

of up to 7.5x10^6 cells/mL could be reached by day 6 in this batch process (clone 4A5). The highest specific productivity, defined as pg amount of recombinant antibody produced by one cell in 24 hours, obtained in this batch process was 10 pg/(cell*day) (pcd) (clone 3C3) (Figure 26 and Table 5).

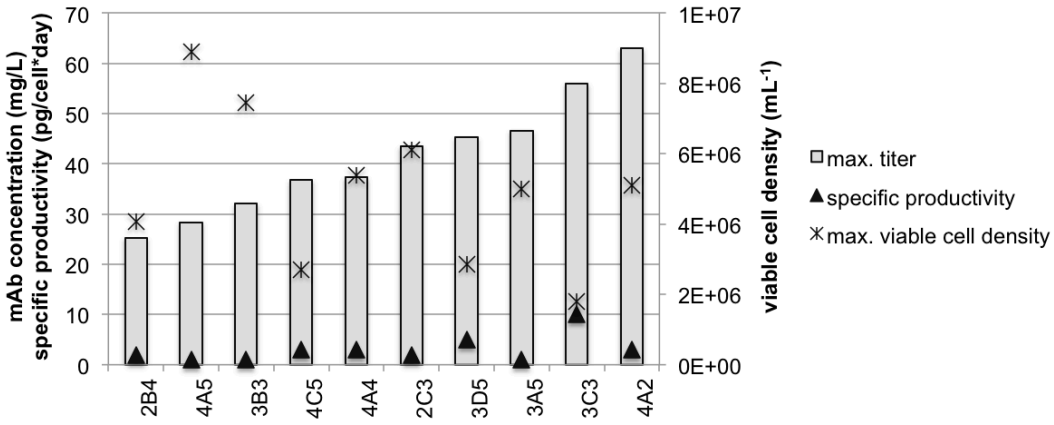

Figure 26: Batch process with anti-CD303-expressing clones isolated by P5CS and zeocin selection
CHO-S clones (of the stable cell line T4.25 pAS-21+55 II) expressing anti-CD303 were selected by P5CS and zeocin and isolated by limiting dilution. Clones were seeded at 2x10^5 cells/mL in 30 mL proline-free CHO MACS CD containing 100 μg/mL zeocin in 125 mL Erlenmeyer flask and were cultivated in an orbital shake incubator. Daily, samples were taken to determine antibody titers and cell numbers to calculate specific productivities.

Table 5: Overview of batch process with limiting dilution clones of stable cell line T4.25 pAS-21+55 II
Batch process with CHO-S clones (of stable cell line T4.25 pAS-21+55 II) expressing anti-CD303 isolated by limiting dilution.

Clone	Max. titer (mg/L)	Max. viable cell density (mL^{-1})	Specific productivity (pcd)	Days of culture before vitality dropped under 80%
2B4	25.30	4.08 x10^6	2	7
4A5	28.40	8.90 x10^6	1	7
3B3	32.13	7.45 x10^6	1	7
4C5	36.79	2.70 x10^6	3	5
4A4	37.35	5.38 x10^6	3	6
2C3	43.53	6.12 x10^6	2	7
3D5	45.26	2.85 x10^6	5	6
3A5	46.59	5.01 x10^6	1	8
3C3	56.06	1.79 x10^6	10	5
4A2	63.00	5.10 x10^6	3	7

To further improve the batch process, different production media containing proline – namely ExCell CD CHO, HyClone SFM4CHO, Gibco FreeStyle CHO, CD FortiCHO and CHO MACS CD – were tested with the two best producing clones T4.25 pAS-21+55 II 3C3 and 4A2. Clones were adapted to the different proline-containing media supplemented with 100 μg/mL zeocin for 4 weeks. The clone 3C3, which showed slow growth and had previously difficulties adapting to the shake incubator, did only survive adaptation to ExCell

CD CHO and HyClone SFM4CHO. For the batch process, zeocin was removed and cells were seeded at $2x10^5$ cells/mL with daily monitoring of antibody concentration in the supernatant and cell counting. As shown in Figure 27 and Table 6, maximum titers of only 18.7 mg/L could be reached. When comparing the growth and production properties of clone 4A2 in the different media, HyClone SFM4CHO led to highest titers, closely followed by CHO MACS CD (8.5% lower). However, the highest viable cell density being 3-fold higher compared to the density in HyClone SFM4CHO could be observed in CHO MACS CD. Cultivation of the clone in the other tested media led to lower titers.

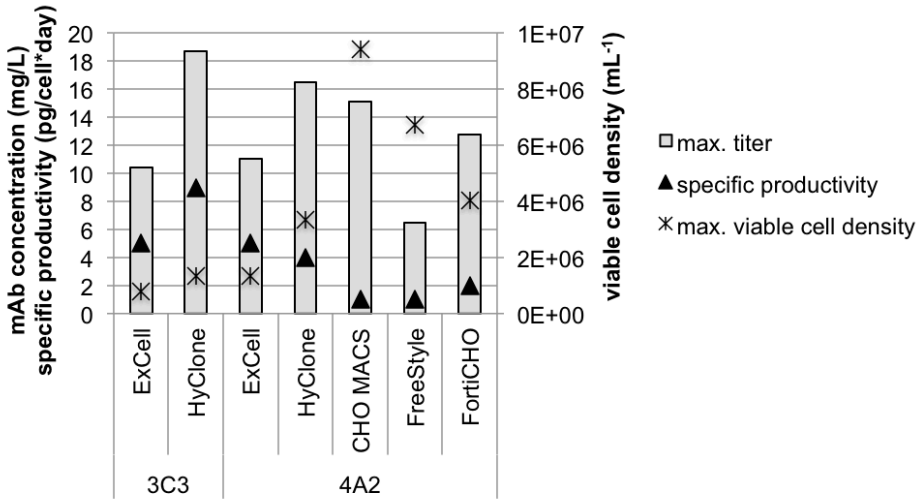

Figure 27: Batch process to test different production media

Two best CHO-S clones (T4.25 pAS-21+55 II 3C3 and 4A2) expressing anti-CD303 selected by P5CS and zeocin were adapted to five different serum-free commercially available production media with proline. Cells were seeded at $2x10^5$ cells/mL in 30 mL of in 125 mL Erlenmeyer flasks and were cultivated in an orbital shake incubator. Daily, samples were taken to determine antibody titers and cell numbers to calculate specific productivities.

Table 6: Overview of batch process with different production media

Batch process with the CHO-S clones 3C3 and 4A2 (of stable cell line T4.25 pAS-21+55 II) expressing anti-CD303 and isolated by limiting dilution in different media.

Clone	Medium	Max. titer (mg/L)	Max. viable cell density (mL^{-1})	Specific productivity (pcd)	Days of culture before vitality dropped under 80%
3C3	ExCell	10.43	$7.72 x10^6$	5	4
	HyClone	18.69	$1.34 x10^6$	9	5
4A2	ExCell	11.03	$1.34 x10^6$	5	4
	HyClone	16.49	$3.33 x10^6$	4	6
	CHO MACS	15.09	$9.44 x10^6$	1	6
	FreeStyle	6.51	$6.72 x10^6$	1	4
	FortiCHO	12.77	$4.04 x10^6$	2	4

3.1.3.1.2 Fed-batch production in suspension shake cultures

The two best producing clones (T4.25 pAS-21+55 II 3C3 and 4A2) of the batch process (Figure 26) were cultivated in a fed-batch process. Cultures for inoculation were maintained in CHO MACS CD medium without proline, containing 100 µg/mL zeocin. For the fed-batch, $2x10^5$ cells/mL were seeded in proline-free CHO MACS CD medium containing 0, 50 or 100 µg/mL zeocin to get first indications about the stability of clones with respect to zeocin. Starting on day 3, cells were fed with CHO MACS Feed Supplement, which containes proline. Thus, the selection pressure of the P5CS selection was removed in all cultures at this point in time. For two to three days, a feed of 3.1% of culture volume was given. Once viable cell densities of over $5x10^6$ cells/mL were reached, the feed was increased to 7.5% for another two days and finally raised to 8% for the remaining culture time. Using a fed-batch process with an antibiotic concentration of 100 µg/mL zeocin, for both clones a final antibody concentration of over 140 mg/L could be reached (Figure 28 and Table 7). At a concentration of 100 µg/mL zeocin, clone 4A2 showed superior growth with a maximum living cell density of $2x10^7$ cells/mL compared to clone 3C3. Clone 3C3 showed lower viable cell densities of maximal $7x10^6$ cells/mL, but a higher specific productivity of 5 pcd (Figure 28 and Table 7). With decreasing zeocin concentrations, the maximum antibody concentrations declined. With 0 µg/mL zeocin, clone 4A2 only produced 75% of the titer at 100 µg/mL and clone 3C3 only 44%. In contrast, the growth and maximum viable cell density was positively influenced by the removal of zeocin being up to 1.6-fold higher (clone 4A2) at 0 µg/mL zeocin concentrations compared to concentrations of 100 µg/mL zeocin.

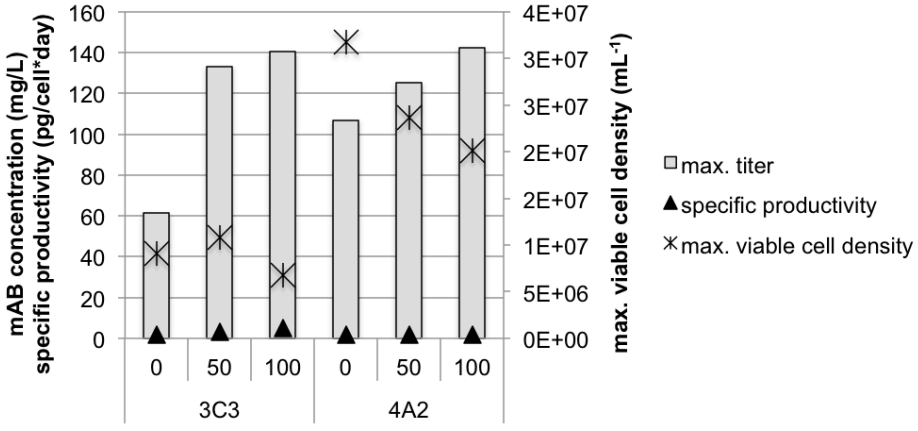

Figure 28: Fed-batch process with anti-CD303-expressing clones isolated by P5CS and zeocin selection

The two best producing clones (T4.25 pAS-21+55 II 3C3 and 4A2) identified in the batch process were cultivated in a fed-batch process: $2x10^5$ cells/mL were seeded in 25 mL CHO MACS CD without proline containing 0, 50 or 100 µg/mL zeocin in 125 mL Erlenmeyer flasks and were cultivated in an orbital shake incubator. Starting on day 3, cultures were fed with CHO MACS Feed Supplement (3% to 8% of culture volume, increased over time). Daily, samples were taken to determine antibody titers and cell numbers to calculate specific productivities.

Table 7: Overview of a fed-batch process of T4.25 pAS-21+55 II limiting dilution clones 3C3 and 4A2
Fed-batch process with the CHO-S clones 3C3 and 4A2 (of stable cell line T4.25 pAS-21+55 II) expressing anti-CD303 isolated by limiting dilution in CHO MACS CD with different zeocin concentrations. Cells were fed with CHO MACS Feed Supplement daily.

Clone	Zeocin concentration (µg/mL)	Max. titer (mg/L)	Max. viable cell density (mL^{-1})	Specific productivity (pcd)	Days of culture before vitality dropped under 80%
3C3	0	61.52	9.10×10^6	2	8
	50	133.18	1.08×10^7	3	8
	100	140.28	6.75×10^6	5	7
4A2	0	106.92	3.17×10^7	2	11
	50	125.20	2.37×10^7	2	11
	100	142.40	2.01×10^7	2	12

These results demonstrate that the isolated P5CS/zeocin-selected clones were suitable for protein expression under production conditions in batch and fed-batch processes.

3.1.3.2 Comparison of different selection markers for the expression of cytokines

To validate the P5CS selection system for the expression of recombinant proteins such as cytokines, the human transforming growth factor beta 1 (hTGF-β1) was chosen as a model protein. To compare the P5CS selection to antibiotic selections, vectors with P5CS as well as resistance genes for neomycin, hygromycin B and zeocin were generated (pJN-1, pJN-2, pJN-3, pJN-4). CHO-S cells were transfected (T6.1/6.2) with these vectors and stable cell lines were generated in selection medium with a reduced dialyzed FBS content of 5%. The hTGF-β1 expression was monitored as a maximum titer, which was determined by seeding the cells in a separate plate in addition to the normal culture and letting these cells overgrow to reach the maximum titer. The hTGF-β1 concentration in the cell culture supernatant was determined by a hTGF-β1-specific ELISA after acid activation. Figure 29 shows the production of hTGF-β1-expressing cell lines generated by the different selection markers. Stable cell lines generated by P5CS selection (T6.1 pJN-1 I and II) and neomycin (T6.2 pJN-2 I and II) or hygromycin B (T6.2 pJN-3 I and II) selection have similar hTGF-β1 production levels of about 17 to 34 ng/mL. In contrast, stable cell lines generated by zeocin selection (T6.1 pJN-4 I and II) produce 78 to 95 ng/mL hTGF-β1, which represents an approximate increase of about 3 to 4-fold.

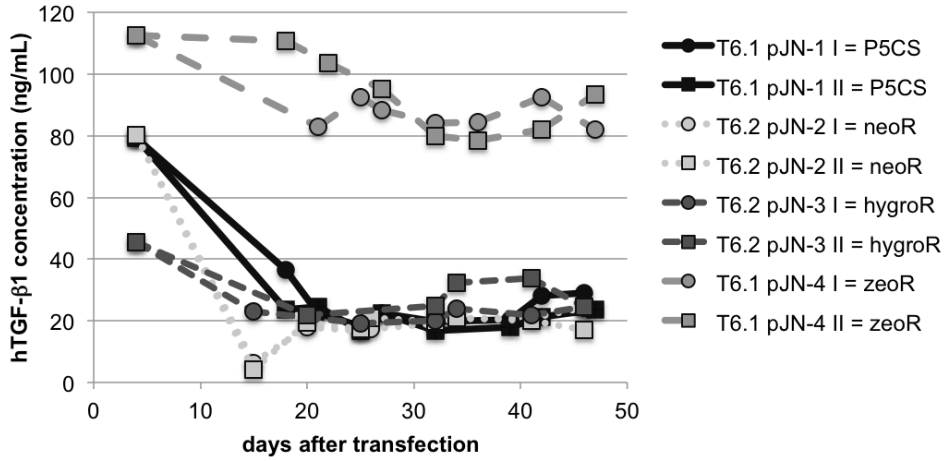

Figure 29: Comparison of different selection markers for the generation of hTGF-β1-expressing cell lines
CHO-S cells were transfected (T6.1/6.2) with pMACS-CHO vectors (pJN-1, pJN-2, pJN-3, pJN-4) containing the cDNA of hTGF-β1 as GOI and P5CS or the resistance gene against neomycin, hygromycin B or zeocin as selection markers. Cells were selected in proline-free CHO-MEM supplemented with 5% dialyzed FBS for P5CS selection or in CHO-MEM supplemented with 5% dialyzed FBS and proline containing selection antibiotics. The hTGF-β1 expression was monitored in supernatants of overgrown cultures by a TGF-β1 specific ELISA.

These results demonstrate that another recombinant protein beside antibodies could be expressed using the P5CS selection system with comparable expression levels to neomycin and hygromycin B selection.

3.2 Development of a novel cell enrichment system

3.2.1 Generation and validation of the tricistronic pMACS-CHO II expression vector

To generate the tricistronic pMACS-CHO-2A expression vector (Figure 30) with coupled expression of GOI and selection marker and an additional reporter protein, a 2A peptide was used. The 2A sequence from the foot and mouth disease virus (FMDV) (=F2A) flanked by two multiple cloning sites was integrated into the second multiple cloning site behind the IRES element of the pMACS-CHO vector. Selection marker genes were PCR-amplified with new restriction enzyme sites and without a stop codon and integrated in frame into the new second multiple cloning site between the IRES element and the 2A peptide sequence. As reporter proteins the fluorescent proteins RFP, GFP or membrane-bound GFP were used. Reporter proteins without the Kozak sequence and the start codon were integrated in frame behind the 2A sequence. This resulted in a fusion ORF of selection marker gene and reporter protein gene coupled by the 2A peptide sequence, which should lead to a cotranslational separation of the two proteins. As a model GOI, cDNAs of the heavy and light chains of the anti-CD303 antibody were integrated into the first multiple cloning sites.

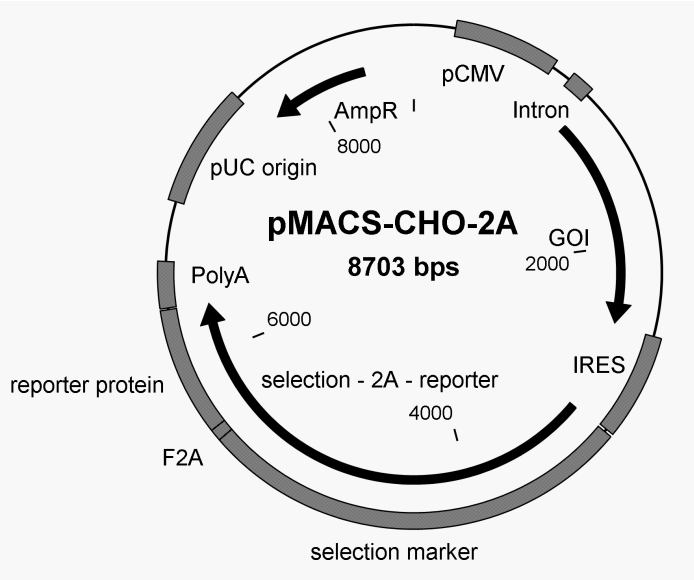

Figure 30: Vector map of the tricistronic pMACS-CHO-2A expression vector
Replacing the second multiple cloning site of the pMACS-CHO vector by a fusion ORF consisting of the selection marker gene, a 2A peptide sequence and a reporter protein generated the tricistronic pMACS-CHO-2A.

Using this tricistronic pMACS-CHO-2A (pAS-83, pAS-84, pAS-85, pAS-86, pAS-107) expression vector for the generation of stable cell lines (T4.28/4.29/4.32/4.33) according to the standard selection protocol revealed several difficulties: 1. The cells did not survive the applied selection pressure, even with reduced antibiotic concentrations (25-50% of usual concentration). 2. Successfully isolated cell lines (T4.32 pAS-84+85, T4.33 pAS-85+108 and T4.33 pAS-86+107), which were identified as double positive in the transient transfection, lost expression of reporter proteins although an antibody titer comparable to cell lines generated with the dicistronic pMACS-CHO vector (T4.20 pAS-21+23 or T4.25 pAS-21+55 I or II) was determined (Figure 31).

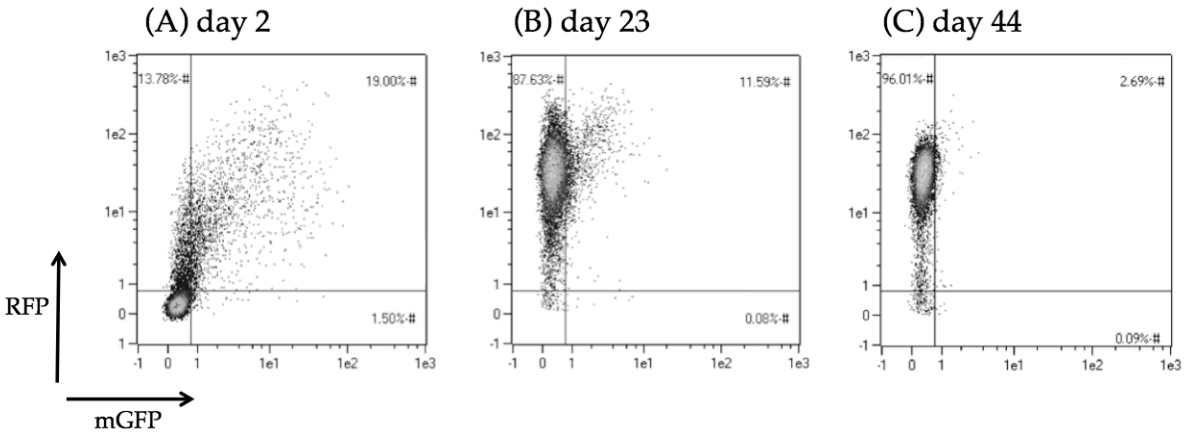

Figure 31: Loss of reporter protein expression in stable cell lines generated with pMACS-CHO-2A vectors
Flow cytometry analysis of a stable CHO-S cell line (T4.33 pAS-86+107 I) cotransfected with the vectors pAS-86 and pAS-107 expressing the anti-CD303 antibody and coexpressing the reporter proteins RFP and a membrane-bound GFP (A) on day 2 (titer 3.0 μg/mL), (B) on day 23 (titer 1.7 μg/mL) and (C) on day 44 (titer 2.5 μg/mL) after transfection.

3. Staining CHO-S cells expressing the membrane-bound GFP, which should localize in the plasma membrane on the cell surface, using a GFP-specific polyclonal antibody and an AlexaFluor647-labeled secondary antibody did not stain any cells transiently transfected with a pMACS-CHO-2A expression vector (pAS-107) (Figure 32). An AlexaFluor647/GFP-double positive population could be detected analyzing CHO-S cells transiently transfected with a positive control vector, the pDisplay-GFP with higher expression levels of the membrane-bound GFP.

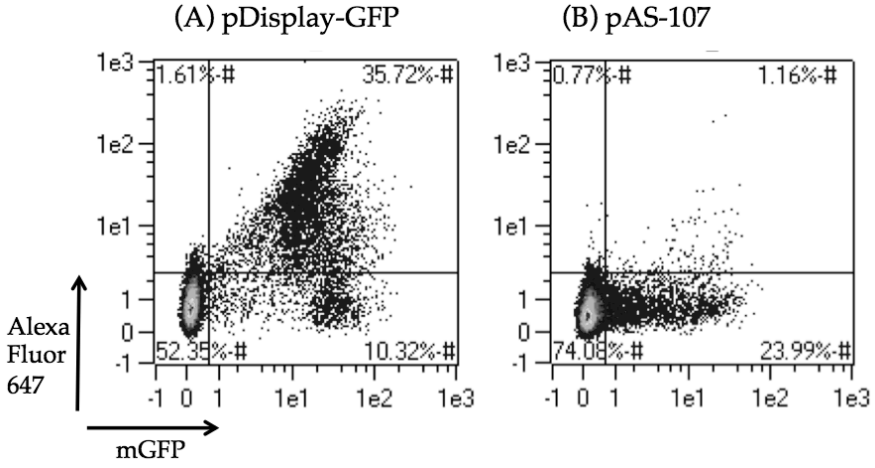

Figure 32: Staining of the membrane-bound GFP of transiently transfected cells
Flow cytometry analysis of a CHO-S cell line transiently transfected with the vectors (A) pDisplay-GFP or (B) pAS-107. The cells were stained with a polyclonal rabbit anti-GFP antibody and a secondary anti-rabbit-AlexaFluor 647 antibody.

In summary, it was concluded that an inefficient cleavage of selection marker and fluorescent protein might be causing the observed problems.

To solve these problems of the pMACS-CHO-2A vector such as inability to stain surface reporter proteins and diminishment of reporter protein expression, the tricistronic cassette was swapped. The F2A sequence was exchanged by the T2A sequence from the *Thosea asigna* virus containing a serine-glycine linker to facilitate improved cleavage. Selection marker genes were PCR-amplified with new restriction enzyme sites without a start codon and Kozak sequence but containing a stop codon and were integrated in frame into the third multiple cloning site. In addition, reporter proteins were PCR-amplified with new restriction enzyme sites without a stop codon but containing a start codon and Kozak sequence and were integrated in frame into the second multiple cloning site resulting in a fusion ORF of reporter protein, 2A peptide sequence and selection marker. Again as a model GOI the heavy and light chain of the anti-CD303 antibody or the hTGF-β1 sequences were integrated into the first multiple cloning site of this pMACS-CHO II vector (Figure 33)

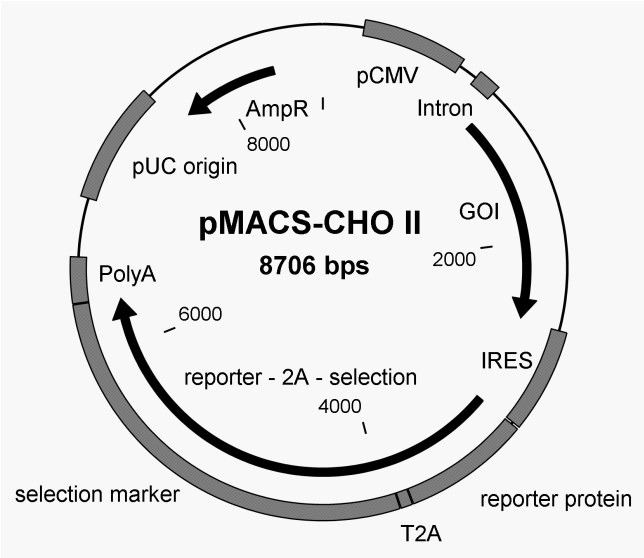

Figure 33: Vector map of tricistronic pMACS-CHO II expression vector

Changing the order of selection marker gene and reporter protein gene of the pMACS-CHO-2A and replacing the F2A peptide sequence by a T2A peptide sequence with serine-glycine linker resulted in the pMACS-CHO II vector.

Using this tricistronic pMACS-CHO II expression vector, stable cell lines could be generated according to the standard selection process. Difficulties surviving the selection process, as seen using the pMACS-CHO-2A vector, could not be observed. As observed for the generation of stable cell lines with the dicistronic pMACS-CHO vector, cells recovered from selection and started expanding again during the first 7 to 10 days after transfection. Expressing anti-CD303 as a model antibody with a combination of P5CS selection for the heavy chain and zeocin selection for the light chain and GFP and RFP as reporter proteins (T4.44 pAS-125+133 II), producer rates of over 90% in less than 18 days can be reached monitored by the GFP/RFP-double positive rate by flow cytometry (Figure 34).

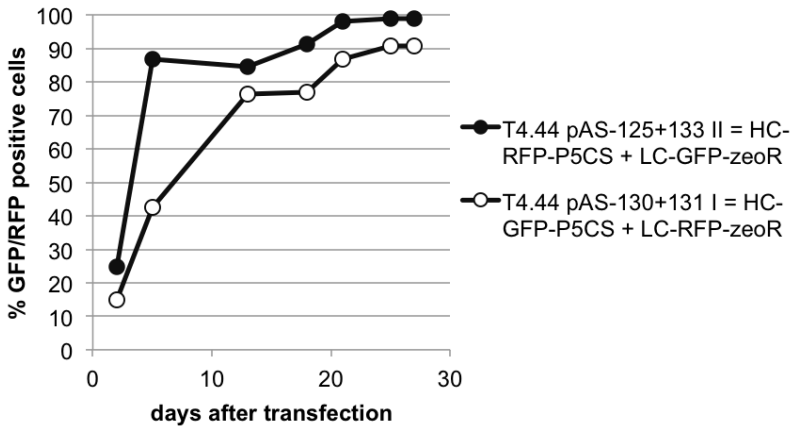

Figure 34: Monitoring of stable CHO-S cell line generated with pMACS-CHO II vectors

Flow cytometry analysis over time of stable CHO-S cell lines (T4.44 125+133 II and pAS-130+131 I) cotransfected with the vectors pAS-125 and pAS-133 (LC-GFP-zeocin + HC-RFP-P5CS) or pAS-130 and pAS-131 (LC-RFP-zeocin + HC-GFP-P5CS).

To ensure that the additional reporter protein in the tricistronic construct has no negative effect on the GOI expression, the di- and tricistronic constructs were compared expressing hTGF-β1 as a model cytokine. As shown in Figure 35, hTGF-β1 produced by stable cell lines generated with the dicistronic vector (T6.1 pJN-1 I and II) or the tricistronic vectors coexpressing GFP (T6.2 pJN-5 I and II) or the membrane-bound (T6.1 pJN-6 I and II) were monitored over time. As usual for the selection process, variations even between cell lines generated with the same vector can be seen. But within these normal variability, the integration of a third gene into the original pMACS-CHO vector does not reduce the hTGF-β1 expression levels.

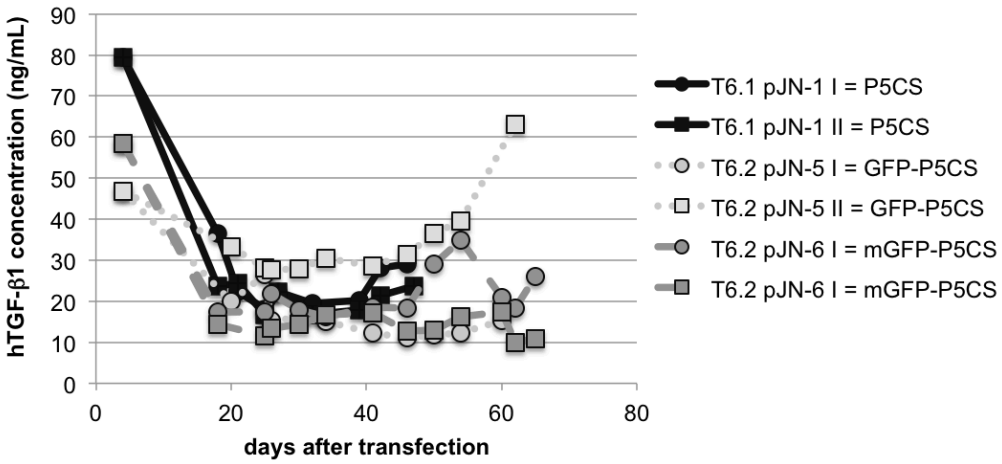

Figure 35: Influence of an additional reporter protein on expression level of the GOI

Expression levels of hTGF-β1 in stable CHO-S cell lines selected by P5CS generated with a dicistronic pMACS-CHO vector (T6.1 pJN-1 I and II) or with tricistronic pMACS-CHO II vectors coexpressing GFP (T6.2 pJN-5 I and II) or a membrane-bound GFP (T6.1 pJN-6 I and II).

Using the improved pMACS-CHO II vector the additional problems with the cell surface staining of the membrane-bound GFP were solved. As shown in Figure 36, staining the membrane-bound GFP with a GFP-specific polyclonal antibody and an AlexaFluor647-labeled secondary antibody led to a correlation between AlexaFluor647 and GFP signal in both pMACS-CHO II vectors, pAS-126 (anti-CD303 LC- mGFP - zeocin) and pAS-132 (anti-CD303 HC- mGFP - P5CS). As a positive control, the pDisplay-GFP vector was used again.

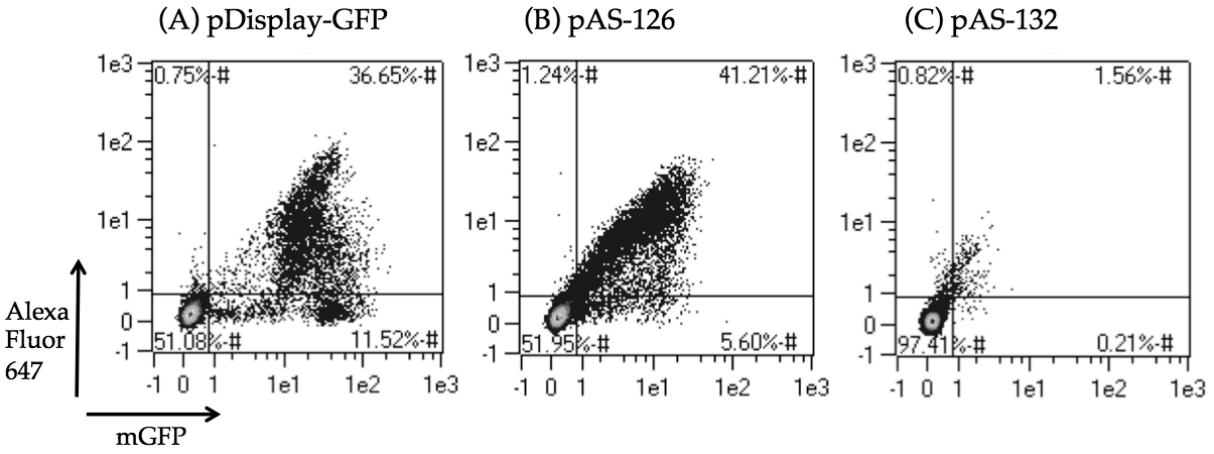

Figure 36: Staining of the membrane-bound GFP of transiently transfected cells

Flow cytometry analysis of a CHO-S cell line transiently transfected with the vectors (A) pDisplay-GFP or (B) pAS-126 (LC-mGFP-zeocin) and (C) pAS-132 (HC-mGFP-P5CS). The cells were stained with a polyclonal rabbit anti-GFP antibody and a secondary anti-rabbit-AlexaFluor 647.

Based on these results, it was concluded that using the improved pMACS-CHO II vector, a correct cleavage of selection marker and fluorescent protein occured and that this tricistronic vector setting did not negatively influence the GOI expression levels.

3.2.2 Clone isolation by FACS sorting

3.2.2.1 Clone isolation by FACS sorting compared to limiting dilution

To validate clone isolation by FACS sorting using the coexpression of the fluorescent reporter proteins, it was compared to a traditional clone isolation method, the limiting dilution. A stable cell line (T4.44 pAS-130+131 I) expressing 1.5 μg/mL anti-CD303 as a model antibody selected by P5CS and zeocin coexpressing GFP and RFP on day 25 after transfection with a producer rate of 93% monitored by GFP/RFP-double positive cells in flow cytometry (Figure 37 A) was used. For FACS sorting, the brightest 0.8% double positive cells (Figure 37 B) were sorted as single cells into five 96-well plates in CHO-MEM containing no proline and no zeocin and an increased dialyzed FBS concentration of 10%. After 5 days of cultivation, medium was added to reach zeocin and FBS concentrations of the usual selection medium (CHO-MEM with glutamine, 10% dialyzed FBS and 100μg/mL zeocin).

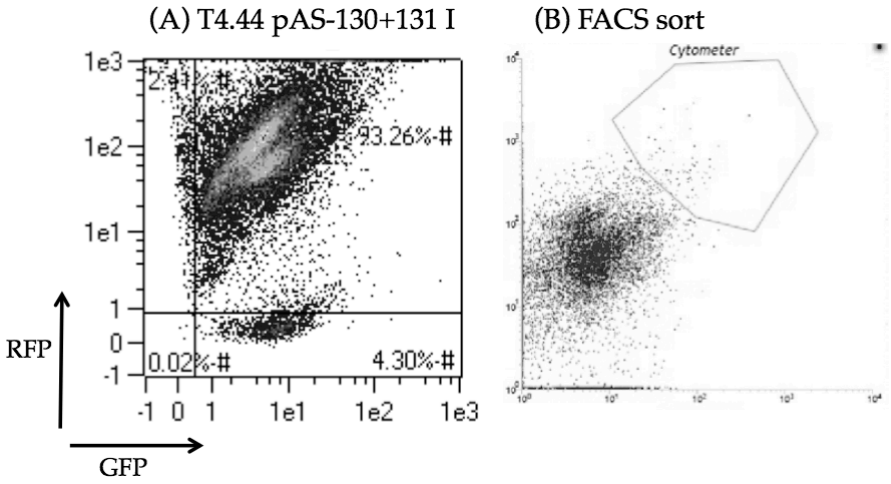

Figure 37: Flow cytometry analysis of an antibody-expressing CHO-S cell line coexpressing RFP and GFP
(A) Flow cytometry analysis of a stable CHO-S cell line (T4.44 pAS-130+131 I) cotransfected with the vectors pAS-130 and pAS-131 expressing the anti-CD303 antibody and coexpressing the reporter proteins RFP and GFP selected by P5CS and zeocin on day 25 after transfection. (B) FACS sorting gate of the same cell line. The brightest 0.8% RFP/GFP-double positive cells were isolated as single cells in 96-well plates.

In parallel, a limiting dilution of the same stable cell line was performed. 0.5 cell was seeded per well of a 96-well plate in the usual selection medium. After 11 days, all 96-well plates were screened for formation of colonies either by microscope for the limiting dilution plates or fluorescence bottom reader for the FACS-sorted plates. The antibody concentrations of the supernatants of wells with a fluorescence signal above the background level or visually containing cells were determined in an ELISA. A maximum antibody concentration of 6.0 μg/mL was detected for clones isolated by limiting dilution and of 40.8 μg/mL for clones isolated by FACS, being 6.8-fold higher (Figure 38).

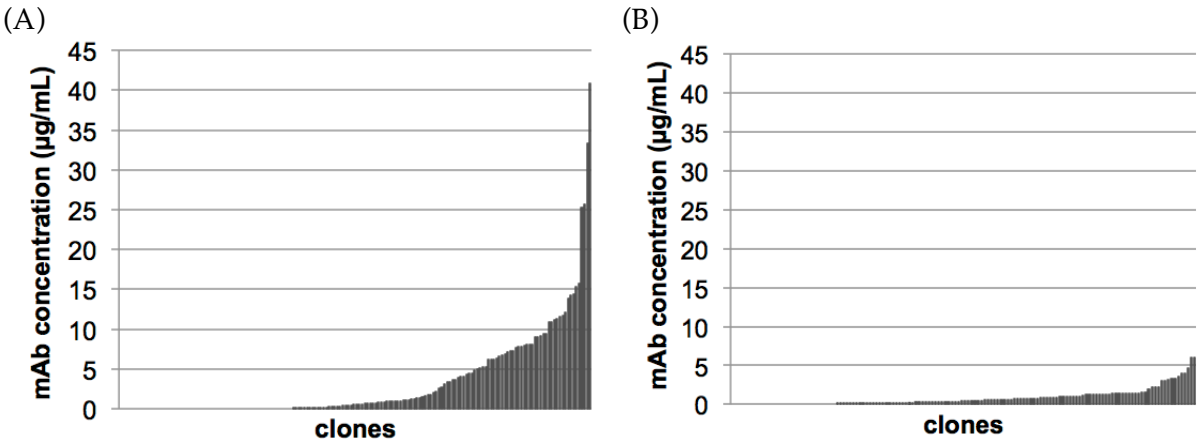

Figure 38: Clone isolation by FACS sorting compared to limiting dilution
CHO-S clones (from T4.44 pAS-130+131 I) expressing anti-CD303 selected by P5CS and zeocin coexpressing the reporter proteins GFP and RFP were either isolated by (A) FACS sorting or (B) limiting dilution. The supernatants of the 96-wells containing a clone were screened by an IgG/kappa-specific ELISA.

The best 24 to 29 of the screened 140 to 170 clones isolated by FACS or limiting dilution were expanded into T75 flasks. Clones were analyzed for cell growth, antibody production in 52h and additionally maximal antibody production by seeding $2x10^5$ cells/well in 12-well plates. In Figure 39, the maximal titer results are shown. The highest producing clone isolated from the stable cell line T4.44 pAS-130+131 I by limiting dilution produced 8.6 μg/mL, which equates to a 5.7-fold increase compared to the starting cell line (Figure 39 B). In comparison, the highest producing clone from the FACS sorting produced an antibody concentration of 15 μg/mL, which equates to a 10-fold increase (Figure 39 A). The average antibody concentration of all expanded clones was 4.3 μg/mL for the limiting dilution clones and 8.1 μg/mL for the FACS-sorted clones (Table 8).

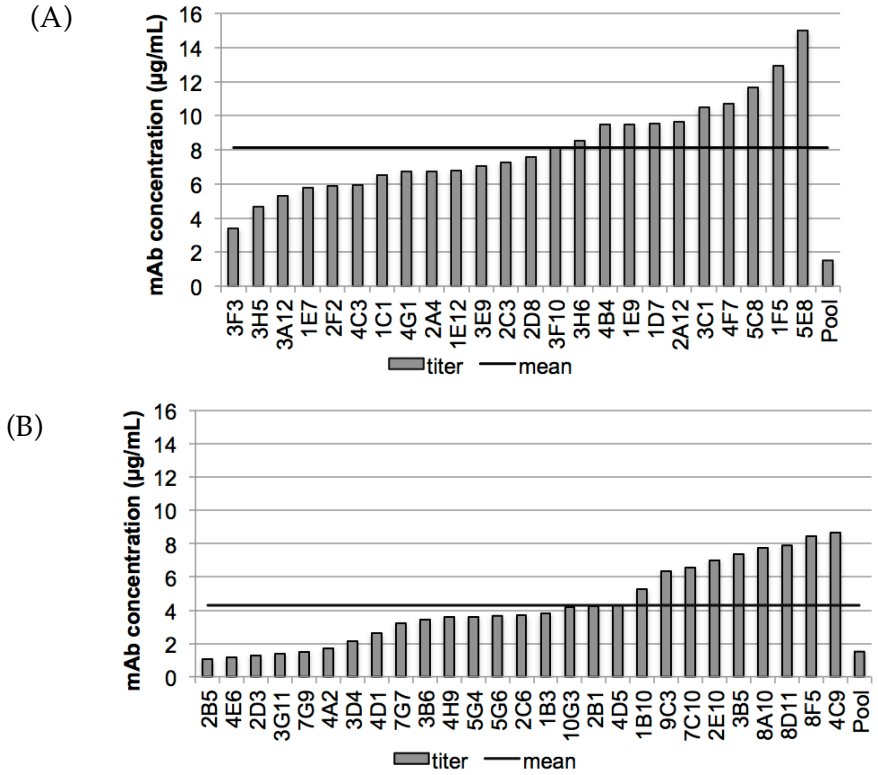

Figure 39: Clone isolation by FACS sorting compared to limiting dilution

CHO-S clones (from stable cell line T4.44 pAS-130+131 I) expressing anti-CD303 selected by P5CS and zeocin coexpressing the reporter proteins GFP and RFP were isolated by (A) FACS sorting or (B) limiting dilution. Best clones of the 96-well screen were expanded. For analysis, $2x10^5$ cells per well of a 12-well plate were seeded and antibody titers of overgrown cultures were determined by ELISA.

Table 8: Clone isolation by FACS sorting compared to limiting dilution

Clones were isolated from the stable cell line T4.44 pAS-130+131 I by limiting dilution or FACS.

	FACS	**Limiting dilution**
Starting mAb concentration (T4.33 pAS-130+131 I) (in static adherent culture)	1.5 μg/mL	1.5 μg/mL
Cloning recovery	35%	9%
max. mAb concentration of best clone after expansion of 24 to 27 best clones (in static adherent culture)	15 μg/mL	8.6 μg/mL
Increase of mAb concentration	10-fold	5.7-fold

In summary, FACS-sorted clones had a 1.7-fold (highest producing clone) to 1.9-fold (mean clone distribution) higher productivity compared to clones isolated by limiting dilution.

3.2.2.2 Multiple FACS subcloning step to isolate high-producers

One of the best clones (5C8) isolated by the first FACS sort (Figure 39) from the cell line T4.33 pAS-130+131 I was subjected to a second subcloning step. The 0.5 % brightest double positive cells were FACS sorted into single cells, the 96-well plates screened and the best 20 clones were expanded as described for the first sorting step. The highest producing clone (1A4) of this second FACS sorting step produced an antibody concentration of 26μg/mL as mamixum titer (Figure 40), which equates to a 3.9-fold increase in the second cloning step; the total titer increase from both cloning steps is 17.3-fold (Table 9). The mean antibody concentration of all 20 expanded clones of the second FACS sorting step was 14 μg/mL.

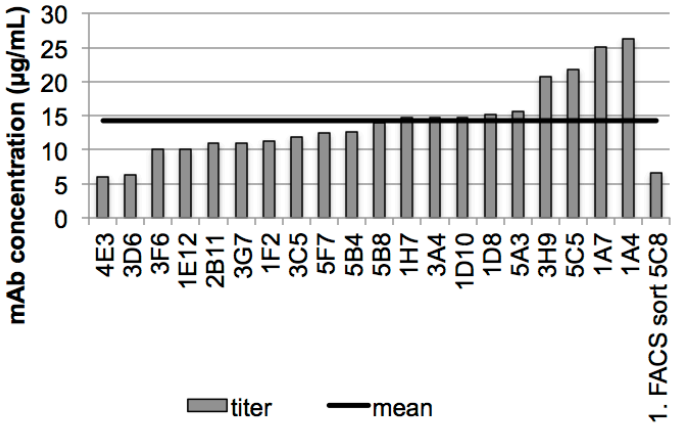

Figure 40: Subcloning by FACS of a FACS-isolated clone

The FACS-isolated clone 5C8 (from the stable cell line T4.44 pAS-130+131 I) expressing anti-CD303 selected by P5CS and zeocin coexpressing the reporter proteins GFP and RFP was subcloned by FACS. The best clones of the 96-well screen were expanded. For analysis 2x10^5 cells per well of a 12-well plate were seeded and antibody titers of overgrown cultures were determined by ELISA.

Table 9: Overview of multiple subcloning steps by FACS for high-producer clone isolation

Clones were isolated from the stable cell line T4.44 pAS-130+131 I by two sequential FACS sorting steps.

	1. FACS sort	**2. FACS sort**
Starting mAb concentration (in static adherent culture)	1.5 μg/mL Pool (T4.33 pAS-130+131 I)	6.6 μg/mL Clone 5C8
Cloning recovery	35%	10%
max. mAb concentration after expansion (in static adherent culture)	11.6 μg/mL Clone 5C8 (third highest)	26 μg/mL Clone 5C8-1A4 (highest producing)
Increase of mAb concentration	7.7-fold	3.9-fold
Inrease of mAb concentration 1.+2. FACS sort	**17.3-fold**	

Comparing these results, it was concluded that using the described FACS sorting strategy it was possible to improve the clone isolation process.

3.2.2.3 Clone isolation by FACS of cytokine-expressing cell line

To verify the universal applicability of the tricistronic vector for clone enrichment using FACS sorting, the clone isolation process was additionally performed with a stable CHO-S cell line (T6.1 pJN-5 I) selected by the P5CS system expressing the cytokine hTGF-β1 as a second model protein and GFP as a reporter protein. As described above for the antibody-producing cell lines, the FACS sort, the 96-well plate screen and the expansion of the best clones were repeated for this hTGF-β1-producing cell line. In the 96-well screen, the highest hTGF-β1 concentration detected was 45.6 ng/mL (data not shown). In the 12-well screen, the highest producing clone of this FACS sort produced a hTGF-β1 concentration of 163 ng/mL (Figure 41), which equates to a 8.8-fold increase in this cloning step from the starting cell line with a maximal cytokine production of 18.5 ng/mL (Figure 35, GFP-P5CS I and Figure 41 Pool). The mean hTGF-β1 concentration produced of all expanded clones was 79 ng/mL (Figure 41).

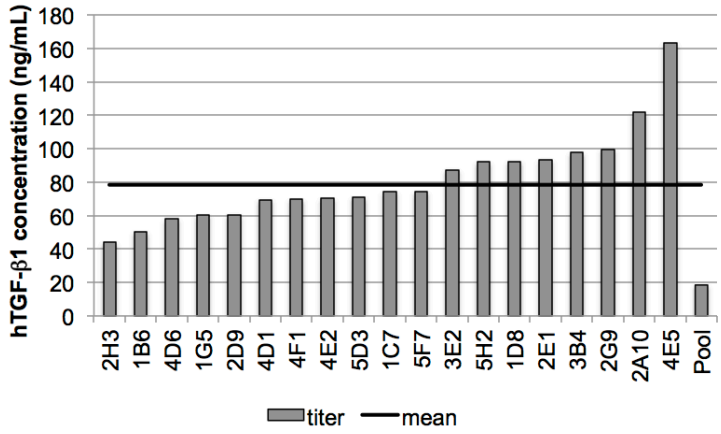

Figure 41: Clone isolation by FACS of a cytokine-expressing cell line

CHO-S clones isolated from the stable cell line T6.1 pJN-5 I expressing hTGF-β1 selected by P5CS coexpressing the reporter protein GFP were isolated by FACS sorting. Best clones of the 96-well screen were expanded. For analysis, $2x10^5$ cells per well of a 12-well plate were seeded and antibody titers of the overgrown cultures were determined by ELISA.

To explore a possible connection of expression levels of the GOI and the fluorescent reporter protein, 16 of the hTGF-β1-expressing clones isolated by FACS from the stable cell line T6.1 pJN-5 I as described above (Figure 41) were analyzed for secreted cytokine concentration, intracellular levels of the cytokine and the MFI of the fluorescent protein. For the intracellular staining of the cytokine, cells were fixed, permeabilized and stained with anti-LAP APC-coupled antibody. LAP binds inactive hTGF-β1. By staining LAP, intracellular

hTGF-β1 levels can be determined. APC fluorescence of fixed and stained cells and GFP fluorescence of non-fixed cells was analyzed by flow cytometry. A TGF-β1-specific ELISA was used to determine secreted hTGF-β1 concentrations in the cell culture supernatants. As shown in Figure 42 A, the intracellular LAP levels, measured as APC fluorescence in the intracellular staining, and GFP fluorescence of the reporter protein showed a strong correlation (Figure 42 A, R^2= 0.8341). In contrast, the hTGF-β1 concentrations and the GFP MFI of the clones did not correlate (Figure 42 B, R^2= 0.0697). This was mainly due to the missing correlation (R^2= 0.0922) of secreted and intracellular cytokine levels as demonstrated in Figure 42 C.

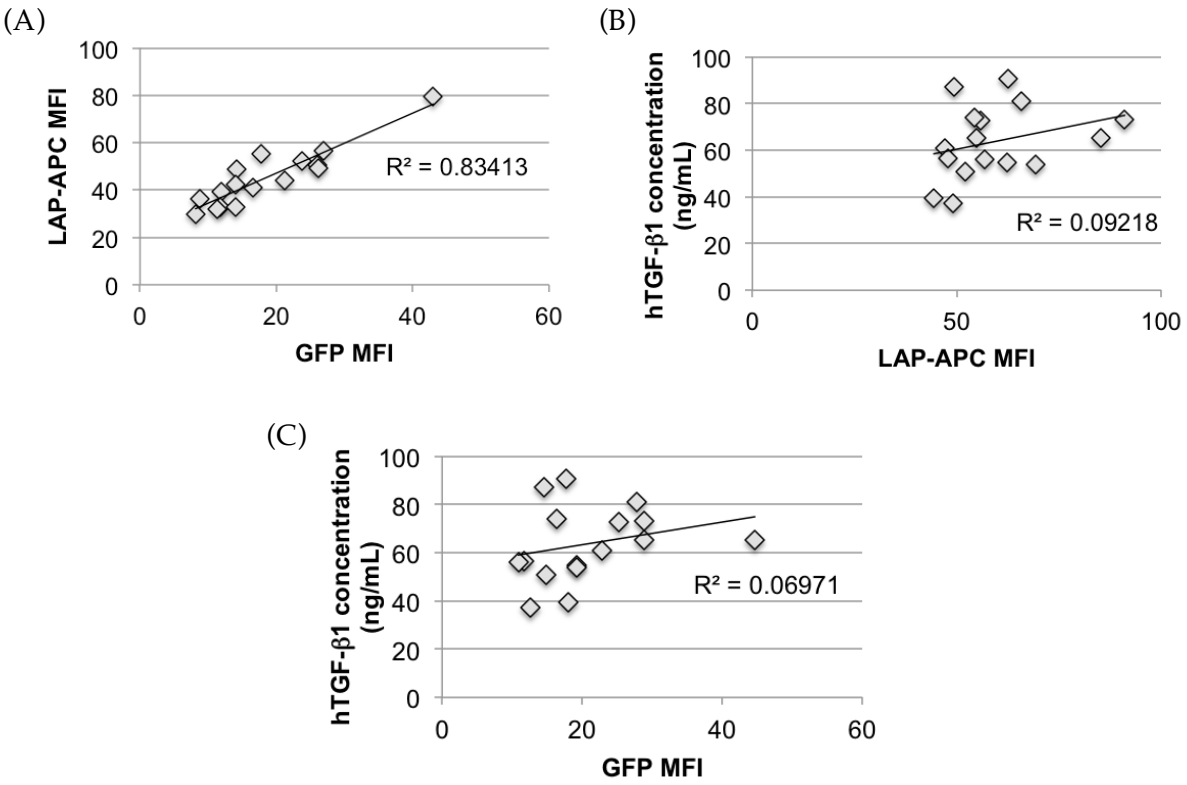

Figure 42: Correlation of cytokine productivity and reporter protein levels

16 CHO-S clones were previously isolated from the stable cell line T6.1 pJN-5 I by FACS sorting expressing hTGF-β1 selected by P5CS coexpressing the reporter proteins GFP. These clones were analyzed regarding intracellular levels of the LAP protein bound to hTGF-β1 as well as GFP reporter protein expression by flow cytometry and the secreted hTGF-β1 in the supernatant by ELISA. (A) Correlation between intracellular LAP and reporter protein, (B) missing correlation between secreted hTGF-β1 in the supernatants and reporter protein, (C) missing correlation between intracellular LAP levels and secreted hTGF-β1.

The ten highest producing clones expressing the hTGF-β1 isolated from the stable cell line T6.1 pJN-5 I by FACS (Figure 41) were adapted to the serum-free production medium CHO MACS CD custom-made without proline. The two best producing clones (4E5 and 2A10) of the 12-well screen could not be adapted to serum-free growth conditions. Already in adherent culture, they showed a more attached phenotype and slow growth. Following the

serum-free adaptation, the remaining 8 clones were incubated in an orbital shake reactor in Erlenmeyer flasks. Some clones showed faster recovery of viability if adapted simultaneously to serum-free conditions and the shake incubator. Once clones recovered to a viability of over 90%, they were subjected to a determination of specific productivities. Therefore, clones were seeded at $2x10^5$ cells/mL in 30 mL medium in 125 mL Erlenmeyer flaks, and an 0.5 mL aliquot was taken daily to measure antibody titers and cell numbers.

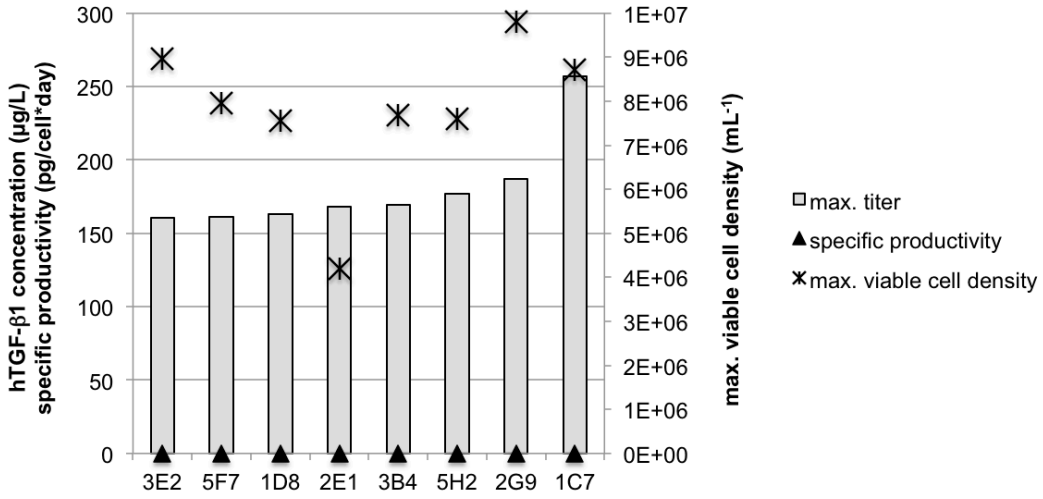

Figure 43: Batch process with hTGF-β1-expressing clones isolated by FACS

CHO-S clones isolated from the stable cell line T6.1 pJN-5 I by FACS sorting expressing hTGF-β1 selected by P5CS and coexpressing GFP were seeded at $2x10^5$ cells/mL in 30 mL CHO MACS CD without proline in 125 mL Erlenmeyer flasks and were cultivated in an orbital shake incubator. Daily, samples were taken to determine antibody titers and cell numbers to calculate specific productivities.

For all analyzed clones, an increase in maximum titer of 1.7-fold to 3.5-fold compared to static, adherent culture condition could be observed. The highest hTGF-β1 concentration reached by clone 1C7 was 257 μg/L. This clone had also the highest specific productivity of 0.03 pcd. Viable cell densities of up to $9.8x10^6$ cells/mL could be reached in this batch process by clone 2G9 (Figure 43 and Table 10). Interestingly, viabilities dropped faster in this batch with hTGF-β1-producing clones compared to anti-CD303-producing clones (from stable cell line T4.25 pAS-21+55 II, compare 3.1.3.1.1). The viability of all hTGF-β1-producing clones was reduced below 80% at the seventh day of batch culture (Table 10). The viability of antibody-producing clones stayed over 80% for up to nine days with most clones having viabilities lower than 80% on day eight of culture (Table 5).

Table 10: Overview of batch process with hTGF-β1-expressing clones isolated by FACS

Batch process with CHO-S clones isolated by FACS from the stable cell line T6.1 pJN-5 I expressing hTGF-β1.

Clone	Max. titer (μg/L)	Max. viable cell density (mL^{-1})	Specific productivity (pcd)	Days of culture before vitality dropped under 80%
3E2	160.7	8.96 $x10^6$	0.01	6
5F7	161.0	7.96 $x10^6$	0.01	6
1D8	163.4	7.56 $x10^6$	0.01	6
2E1	168.4	4.20 $x10^6$	0.02	5
3B4	169.7	7.68 $x10^6$	0.01	6
5H2	177.0	7.60 $x10^6$	0.01	6
2G9	186.8	9.80 $x10^6$	0.02	6
1C7	256.8	8.72 $x10^6$	0.03	6

The nine highest hTGF-β1-producing clones isolated from the stable cell line T6.1 pJN-5 I by FACS sorting (Figure 41) also used for the batch production were tested regarding their stability in proline-containing medium. Clones were cultivated in parallel in CHO-MEM supplemented with 5 % dialyzed FBS with or without proline and hTGF-β1 titers of supernatants of overgrown cultures were analyzed by an hTGF-β1-specific ELISA. Comparing the titers in the medium with and without proline after 17 days revealed that titers in proline-containing medium were 13 % lower to 16 % higher compared to the ones from proline-free media (Figure 44 A). After 40 days, all clones showed a reduced productivity in proline-containing medium. Titer decreases ranged from 1% to 42% (Figure 44 B).

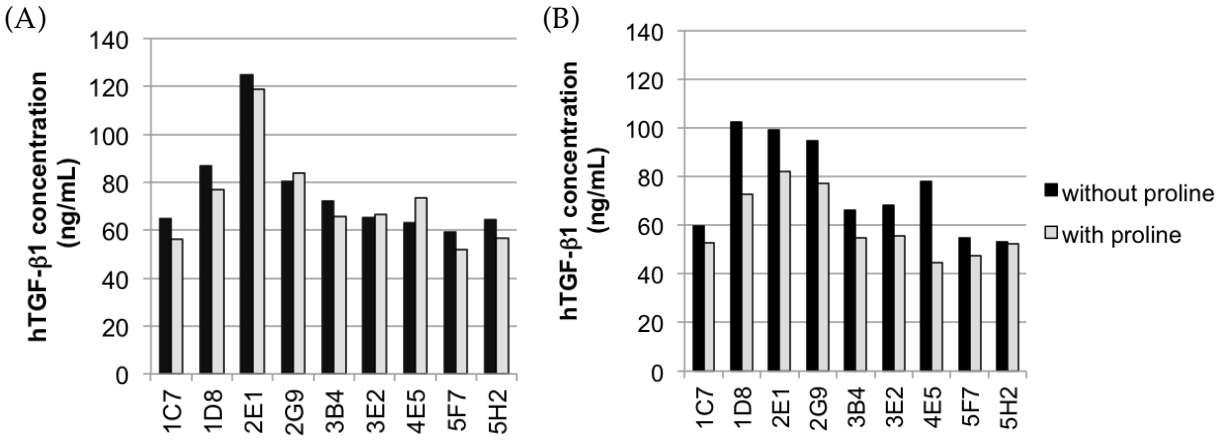

Figure 44: Stability of P5CS-selected hTGF-β1-expressing clones

Nine CHO-S clones isolated from the stable cell line T6.1 pJN-5 I by FACS sorting expressing hTGF-β1 selected by P5CS and coexpressing GFP were cultivated both in CHO-MEM with proline or without proline. After (A) 17 days and (B) 40 days hTGF-β1 concentrations in the supernatants of overgrown cultures were determined.

From these data it was concluded that FACS-isolated clones showed a correlation between intracellular recombinant protein levels and reporter protein fluorescence, which might

explain the enrichment during FACS sorting. These FACS-isolated clones were stable in medium without selection pressure for a certain time frame.

3.2.3 High-producer enrichment by MACS

The coexpression of reporter proteins can additionally be used for enrichment by the MACS technology. For this, at least one reporter protein needs to be membrane-bound. To validate high-producer enrichment using MACS technology, a stable cell line (T4.44 pAS-126+133 II) expressing the anti-CD303 model antibody selected by P5CS and zeocin coexpressing RFP and membrane-bound GFP was generated. The starting producer cell line expressed about 0.7 to 2.0 μg/mL antibody (data not shown). For the MACS enrichment, the membrane-bound GFP was stained indirectly with a polyclonal rabbit anti-GFP antibody followed by a monoclonal anti-rabbit microbead-coupled antibody. Cells were MACS enriched using a MS column. The enriched cells were cultivated in the usual selection medium and expanded for the next MACS enrichment. For the first and second MACS enrichment, cells were separated using only one column. For the third and forth enrichment, the more stringent protocol using two sequential columns was used. The enrichment rate was monitored as GFP fluorescence by flow cytometry and additionally the maximum antibody concentration in the supernatant was monitored. As an example, the percentage of GFP-expressing cells could be increased from 50% (Figure 45 A) to 95% (Figure 45 B) in the first enrichment step of the described stable cell line. Due to the low expression of GFP in cell lines due to the tricistronic pMACS-CHO II vector setting, only cells expressing high levels of GFP are enriched, which is demonstrated by an increase of MFI from 2.5 to 5.2 (Figure 45 A and Figure 45 B).

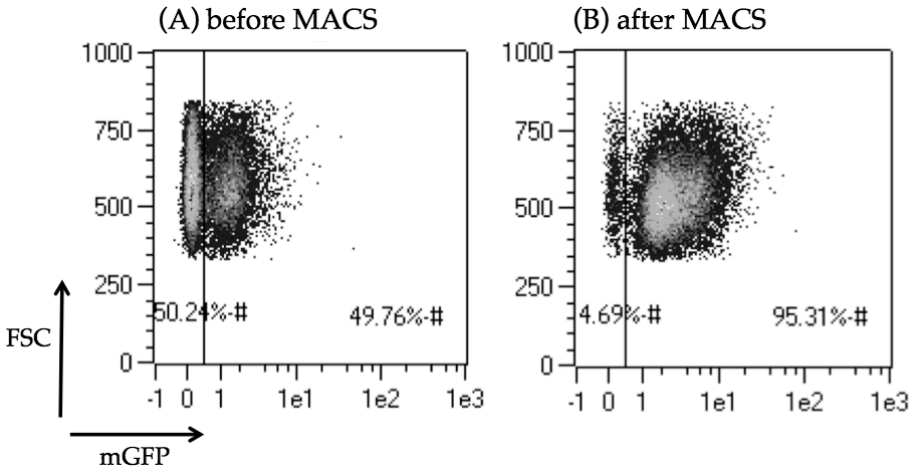

Figure 45: MACS enrichment of an antibody-producing cell line

A Stable CHO-S cell line (T4.44 pAS-126+133 II) expressing the anti-CD303 antibody selected by P5CS and zeocin and coexpressing RFP and a membrane-bound GFP was stained with a polyclonal rabbit anti-GFP antibody and a secondary anti-rabbit antibody conjugated to microbeads and enriched by MACS technology using a MS column. GFP fluorescence was monitored by flow cytometry (A) before and (B) after enrichment.

Since both, the starting cell line as well as the MACS-enriched cell line, showed the usual titer variations over culture time, the titer increase could only be estimated. Due to the coupled expression of GOI and mGFP, it was possible to increase the produced antibody concentration about 2-fold looking at the more constant titer levels after the initial higher increase (Figure 46 B: stable cell line T4.44 pAS-126+133 II to MACS 1). Also in the following enrichment steps, the number of GFP-positive cells (Figure 46 A) and the antibody titer (Figure 46 B) could further be improved. The final four times enriched production cell line (Figure 46, MACS 4) produced an antibody concentration of 8 $\mu g/mL$, which decreased to 5 $\mu g/mL$ during the following 40 days of culture. This resulted in an initial increase of 8-fold reducing to an about 4.5-fold enrichment compared to the starting cell line.

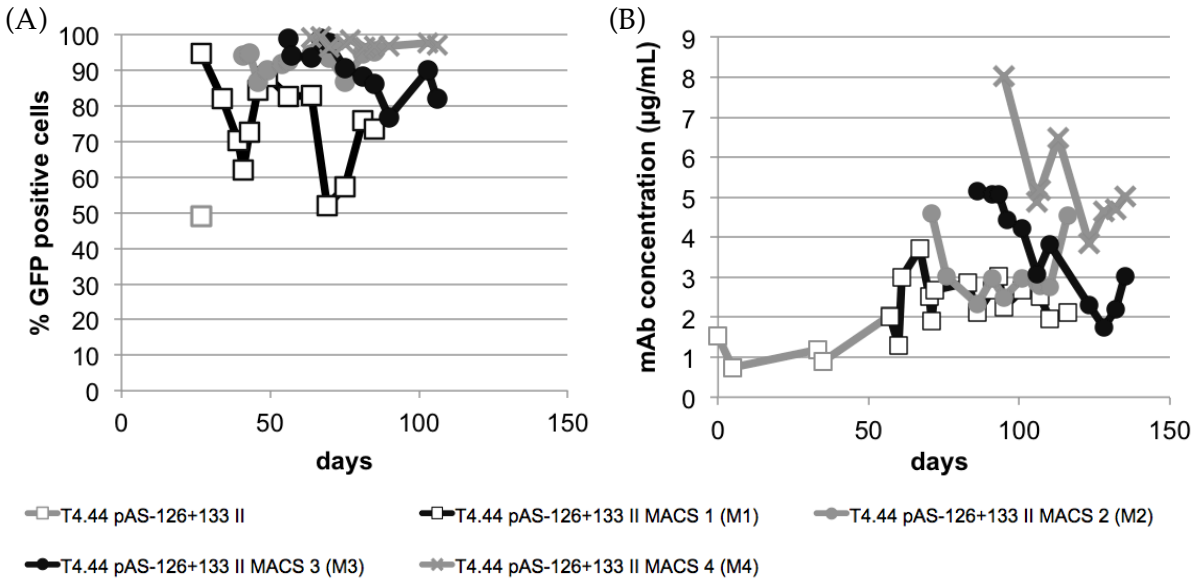

Figure 46: Repeated MACS enrichment of an antibody-producing cell line
A stable CHO-S cell line (T4.44 pAS-126+133 II) expressing the anti-CD303 antibody selected by P5CS and zeocin and coexpressing RFP and a membrane-bound GFP was repeatedly enriched four times by MACS technology. The enriched cell lines were monitored for (A) GFP fluorescence by flow cytometry and (B) antibody concentration in the supernatants by ELISA.

To verify the universal applicability of the MACS enrichment, the process was repeated with a stable CHO-S cell line (T6.2 pJN-6 I) expressing the human cytokine hTGF-β1. The cell line coexpressed the membrane-bound GFP as a reporter protein and was selected by the P5CS system (Figure 35, mGFP-P5CS I). Due to a lower expression level of the membrane-bound GFP in combination with P5CS as a selection marker compared to zeocin (Figure 36), two different enrichment strategies were compared using either a monoclonal direct labeling or a polyclonal indirect labeling. As shown in Figure 47 A, the starting producer cell line contained about 45% GFP-positive cells. Enrichment with the polyclonal indirect strategy led to an increase to 66% GFP-positive cells (Figure 47 B) and an increase of GFP MFI from 0.77 to 2.18, but with a very low cell recovery rate of less than 0.1% for the GFP-positive cells

(data not shown). The expanded cells did not show any improved productivity compared to the starting cell line (Figure 48) and were discarded after a few passages.

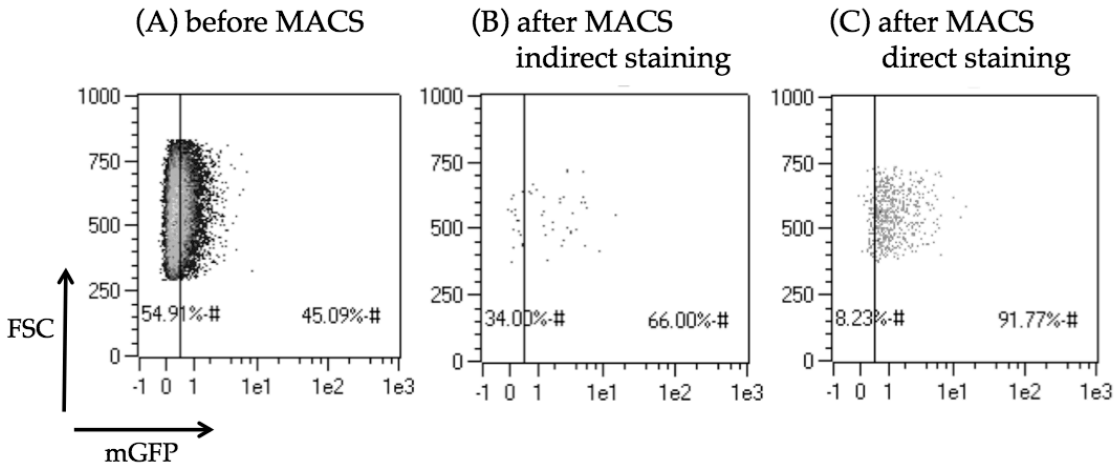

Figure 47: MACS enrichment of a cytokine-producing cell line

A stable CHO-S cell line (T6.1 pJN-6 I) expressing hTGF-β1 selected by P5CS and coexpressing a membrane-bound GFP was labeled with microbeads using a polyclonal indirect anti-GFP labeling or a monoclonal direct labeling. GFP fluorescence was monitored by flow cytometry (A) before and after enriched by MACS technology using a MS column with (B) polyclonal indirect or (C) monoclonal direct staining. For the polyclonal indirect as well as the monoclonal direct staining the same volumes of enriched cell fractions were measured.

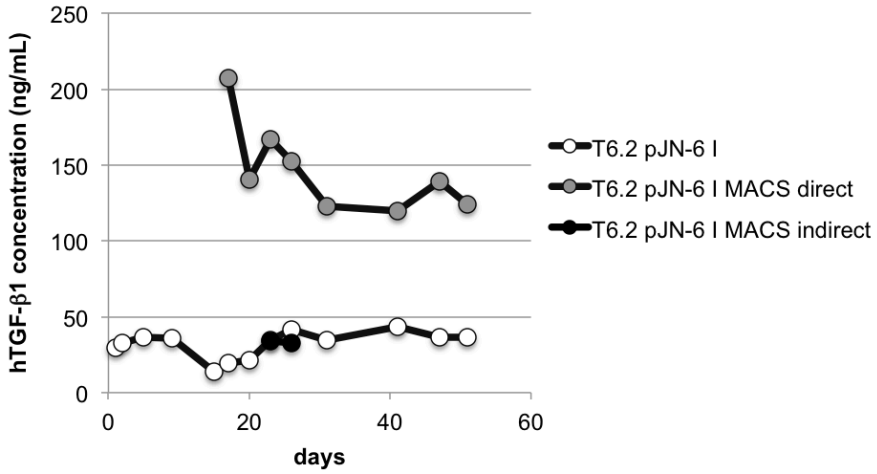

Figure 48: MACS enrichment of a cytokine-producing cell line

A stable CHO-S cell line (T6.1 pJN-6 I) expressing hTGF-β1 selected by P5CS and coexpressing a membrane-bound GFP was labeled with microbeads using a polyclonal indirect anti-GFP staining or a monoclonal direct staining. The secreted hTGF-β1 levels were measured by ELISA.

Using the monoclonal direct strategy, it was possible to enrich GFP-positive cells from 45% to 92% (Figure 47 C) and increase the GFP MFI from 0.77 to 1.55 with a recovery rate of GFP-positive cells of over 2% (data not shown). The monitored hTGF-β1 concentrations improved from about 30 ng/mL before to over 200 ng/mL after the monoclonal MACS enrichment (Figure 48). These levels are comparable to FACS-enriched clones (Figure 41). During the

following passages, the hTGF-β1 production dropped to about 120 ng/mL but remained stable at this level for the following 25 days of culture. This still corresponds to a titer increase of about 4-fold compared to the starting cell line.

To characterize the difference between the monoclonal direct and polyclonal indirect staining, cells were additionally stained with the labeling check reagent-APC, which specifically binds to microbeads. As shown in Figure 49, both staining strategies specifically stain the membrane-bound GFP-positive cells and lead to similar GFP/APC-double positive rates of 86%. A correlation between APC and GFP MFI is visible. High GFP-positive cells are also high APC-positive with the exception of one APC-dim population. The important difference between both staining strategies is the mean APC fluorescence. The monoclonal direct staining (APC MFI 77.4) leads to an APC MFI about 3 times higher compared to the polyclonal indirect staining (APC MFI 26.6).

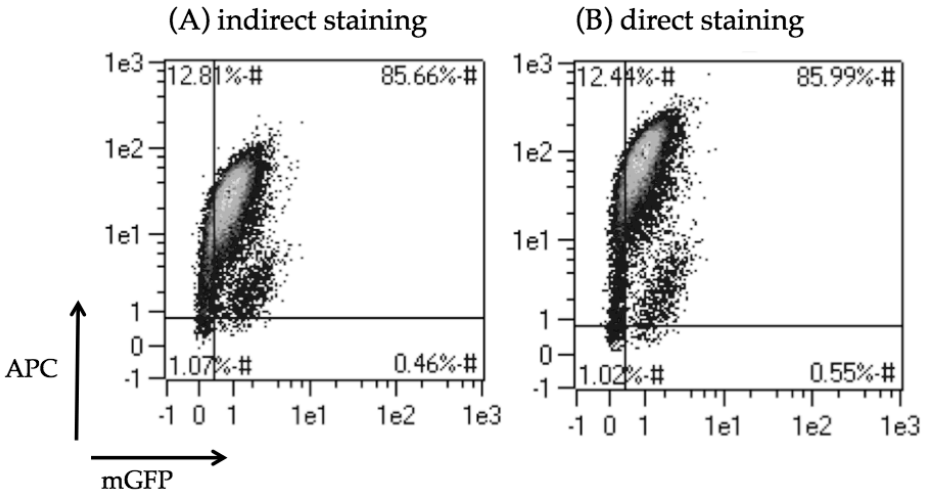

Figure 49: Labeling check of direct and indirect stained cells

A stable CHO-S cell line (T6.1 pJN-6 I) expressing hTGF-β1 selected by P5CS and coexpressing a membrane-bound GFP was labeled with microbeads using a (A) polyclonal indirect anti-GFP staining or a (B) monoclonal direct staining. Staining was controlled using the labeling check reagent-APC. GFP and APC fluorescence were monitored by flow cytometry.

From these results it was concluded that MACS enrichment might be a potent and fast method to improve stable cell lines.

3.2.3.1 Clone isolation by FACS of a MACS pre-enriched cell line

To test a combination of a MACS pre-enrichment and a FACS clone isolation process, the four times enriched stable cell line T4.44 pAS-126+133 II M4 cell lines described in Figure 46 (MACS 4) was FACS sorted as described in 3.2.2.1. The 0.5% best GFP/RFP-double positive cells were sorted as single cells into 5x 96-well plates. Fluorescence bottom reader and microscope were used to identify the 122 wells containing clones. The supernatants of these

wells were analyzed by ELISA. Already 8 days after FACS sorting, the highest titer detected in the 96-well plate screen was 2.0 $\mu g/mL$. The 19 highest expressing clones were expanded and analyzed by seeding $2x10^5$ cells/well of a 12-well plate. Analysis of cell growth and antibody production in 52h and additionally maximally produced antibody concentrations were performed. The mean maximal antibody concentration of these clones was 16 $\mu g/mL$. The highest maximal antibody concentration amounted to 23 $\mu g/mL$, which equates to a 4.6-fold increase in the FACS cloning step (Figure 50). The total increase of antibody production from MACS enrichment and FACS sort is 20.9 (Table 11), which is similar to the results obtained from the two repeated FACS sorting steps described in 3.2.2.2.

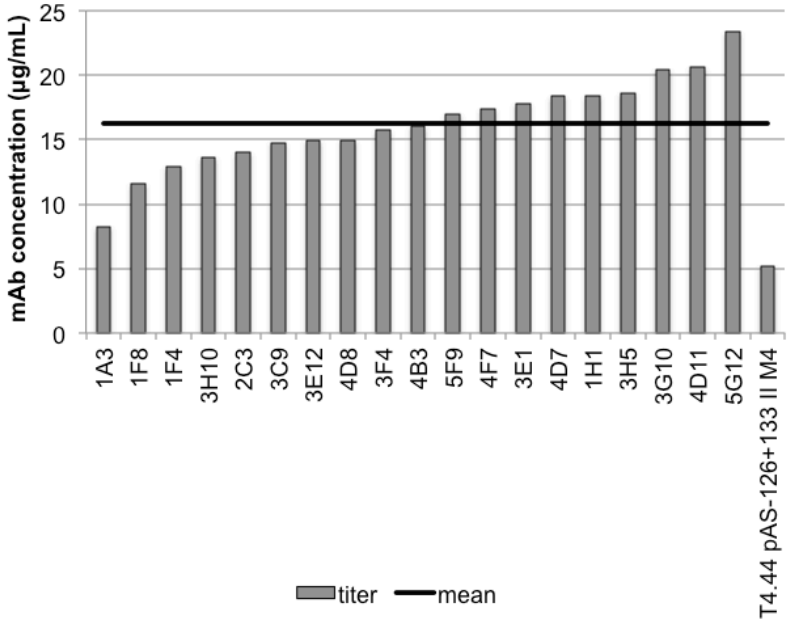

Figure 50: Clone isolation by FACS of a MACS pre-enriched cell line

A MACS pre-enriched cell line (T4.44 pAS-126+133 II M4) expressing anti-CD303 selected by P5CS and zeocin coexpressing the reporter proteins mGFP and RFP was subcloned by FACS. Best 19 clones of the 96-well screen were expanded. For analysis, $2x10^5$ cells per well were seeded in 12-well plates and antibody titers of supernatants of overgrown cultures were determined by ELISA.

Table 11: Overview of clone isolation by FACS of a MACS pre-enriched cell line

The stable cell line T4.44 pAS-126+133 II was MACS-enriched and clones were isolated by FACS.

	MACS enrichment	**FACS sort**
Starting mAb concentration (in static adherent culture)	1.1 $\mu g/mL$ T4.44 pAS-126+133 II	5 $\mu g/mL$ T4.44 pAS-126+133 II M4
Cloning recovery	-	25%
mAb concentration after expansion of 20 best clones (in static adherent culture)	5 $\mu g/mL$ T4.44 pAS-126+133 II M4	23 $\mu g/mL$ T4.44 pAS-126+133 II M4 Clone 5G12
Increase of mAb concentration	4.5-fold	4.6-fold
Increase of mAb concentration: MACS enrichment + FACS sort	20.9-fold	

To test productivity in a batch process, the starting stable cell line (Pool= T4.44 pAS-126+133 II), the four times MACS-enriched stable cell line (M4 = T4.44 pAS-126+133 II M4) and the eight highest producing clones isolated by FACS were used. Cells were adapted to serum-free conditions and cultivation in an orbital shake incubator. The batch process was performed as described in 3.1.3.1.1: $2x10^5$ cells/mL were seeded in 30 mL proline-free CHO MACS CD with 100 μg/mL zeocin. Each day an aliquot was taken to measure antibody titers and cell numbers.

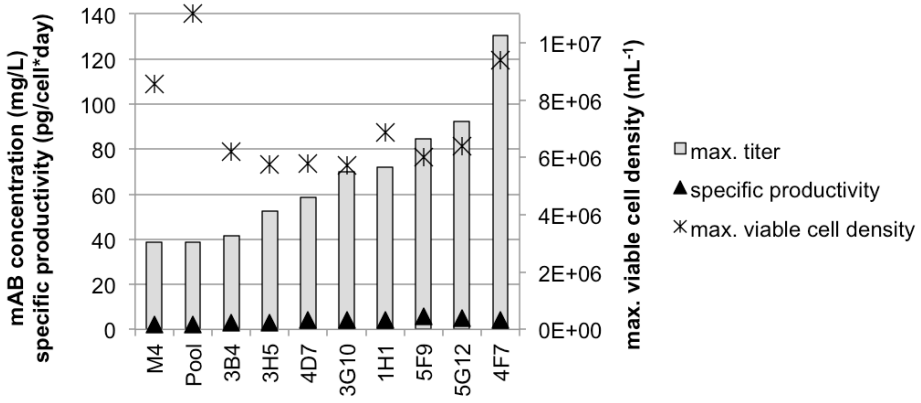

Figure 51: Batch process with clones isolated by MACS and FACS

CHO-S clones isolated from a MACS-enriched stable cell line (T4.44 pAS-126+133 II M4) expressing anti-CD303 selected by P5CS and zeocin coexpressing RFP and the membrane-bound GFP. The starting cell line (Pool= T4.44 pAS-126+133 II), a four time MACS pre-enriched cell line (M4= T4.44 pAS-126+133 II M4) and eight clones isolated by FACS from the MACS pre-enriched stable cell line were seeded at $2x10^5$ cells/mL in 30 mL CHO MACS CD without proline containing 100 μg/mL zeocin in 125 mL Erlenmeyer flasks and were cultivated in an orbital shake incubator. Daily, samples were taken to determine antibody titers and cell numbers to calculate specific productivities.

Table 12: Overview of batch process with clones isolated by MACS and FACS

Batch process with the stable cell line T4.44 pAS-126+133 II, the MACS pre-enriched cell lines T4.44 pAS-126+133 II M4 and FACS-isolated clones from this enriched cell line.

Cell line or clone	Max. titer (μg/L)	Max. viable cell density (mL^{-1})	Specific productivity (pcd)	Days of culture before vitality dropped under 80%
T4.44 pAS-126+133 II M4	38.58	8.57 $x10^6$	2	8
T4.44 pAS-126+133 II	38.82	11.3 $x10^6$	2	7
3B4	41.71	6.20 $x10^6$	3	7
3H5	52.38	5.76 $x10^6$	3	7
4D7	58.69	5.80 $x10^6$	4	7
3G10	70.01	5.72 $x10^6$	4	7
1H1	72.03	6.88 $x10^6$	4	7
5F9	84.56	6.00 $x10^6$	6	6
5G12	92.37	6.40 $x10^6$	5	7
4F7	130.42	9.40 $x10^6$	4	9

For all analyzed clones, an increase in maximum titers of 2.6-fold to 7.5-fold compared to static, adherent culture condition could be observed (Figure 50). The highest antibody concentration could be reached by clone 4F7 with 130 mg/L and the highest specific productivity was reached by clone 5F9 with 6 pcd (Figure 51 and Table 12). Viable cell densities of up to $1.1x10^7$ cells/mL could be reached by the starting stable cell line (Figure 51 Pool).

Based on the obtained results, it was concluded that a combination of MACS enrichment and FACS clone isolation might be very potent method to isolate high producing clones, which are suitable for production in a batch process.

3.2.3.2 Correlation of fluorescent protein and recombinant antibody expression

Futher experiments were dedicated to analyze whether a correlation between expression of one of the fluorescent reporter proteins and the recombinant antibody secretion exists. Therefore, different clones expressing the anti-CD303 antibody generated by a two-vector strategy were analyzed. As shown in Figure 52 A, there exists no correlation between cytoplasmic GFP and antibody expression in 14 clones isolated from the T4.44 pAS-130+131 I cell line by two FACS cloning steps as described in 3.2.2.2. Analyzing 14 clones from the MACS pre-enriched T4.44 pAS-130+131 I M4 cell lines isolated by FACS cloning as described in 3.2.3.1 reveals a correlation between membrane-bound GFP and antibody expression (R^2= 0.50) as shown in Figure 52 B.

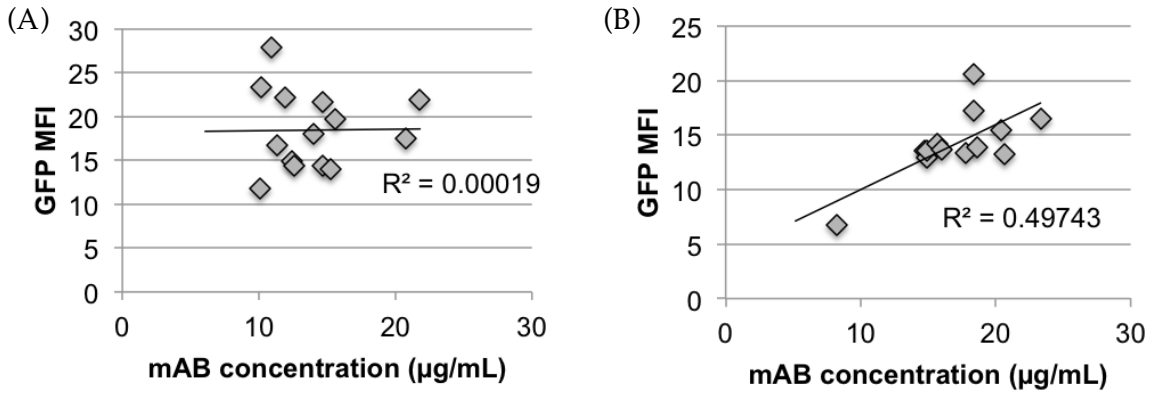

Figure 52: Analysis of correlation between fluorescent protein and recombinant antibody expression
Analysis of fluorescent protein by flow cytometry and recombinant antibody expression by ELISA of (A) highest producing clones isolated by two FACS cloning steps from the T4.44 pAS-130+131 I cell line coexpressing RFP and GFP and (B) clones isolated from the T4.44 pAS-126+133 II M4 four times MACS pre-enriched cell line followed by FACS cloning coexpressing RFP and membrane-bound GFP.

These results indicate that using a membrane-bound fluorescent protein in combination with MACS might increases a correlation of GOI and reporter protein expression.

3.2.4 Combination with an amplifiable selection system

To test a combination of P5CS and zeocin selection with an additional amplification step, the DHFR gene with a separate SV40 promoter was integrated into the tricistronic vector pAS-130 encoding the anti-CD303 light chain, the zeocin resistance gene and RFP to obtain the vector pAS-158 (Figure 53).

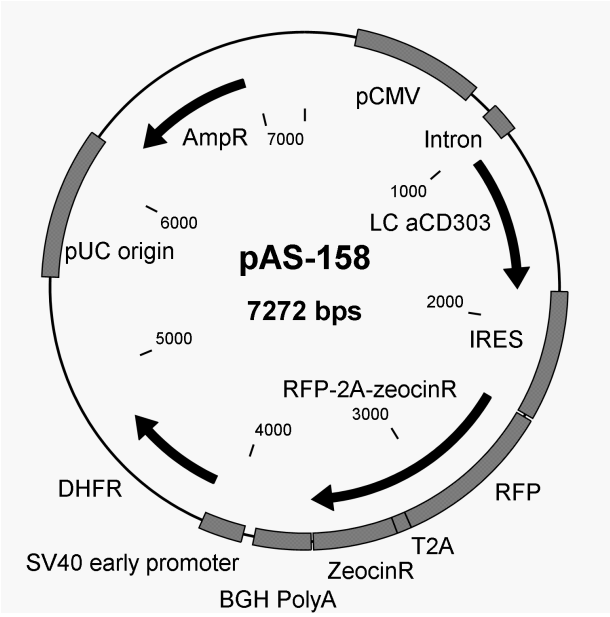

Figure 53: pMACS-CHO II vector with additional DHRF gene

The DHFR gene controlled by a separated SV40 promoter was added into the pAS-130 vector.

The vectors pAS-131 and pAS-158 were cotransfected into CHO-S (T4.50) and first selected with the usual selection medium for P5CS and zeocin selection (CHO-MEM without proline, with 100 ng/mL zeocin and 5% dialyzed FBS). Once stable cell lines (T4.50 pAS-131+158 I and II) were established, 100 or 200 nM MTX were added. These MTX concentrations were more challenging than a usual selection process. Many cell lines did not recover. The ones that did recover had selection periods of over 3 months and showed reduced growth properties after recovery. Antibody titers on day 105 after transfection show that the MTX treatment did not increase the productivity. Stable cell lines treated with 100 or 200 nM MTX produced about 30-fold less antibody than the starting cell line T4.50 pAS-131+158 I as shown in Figure 54.

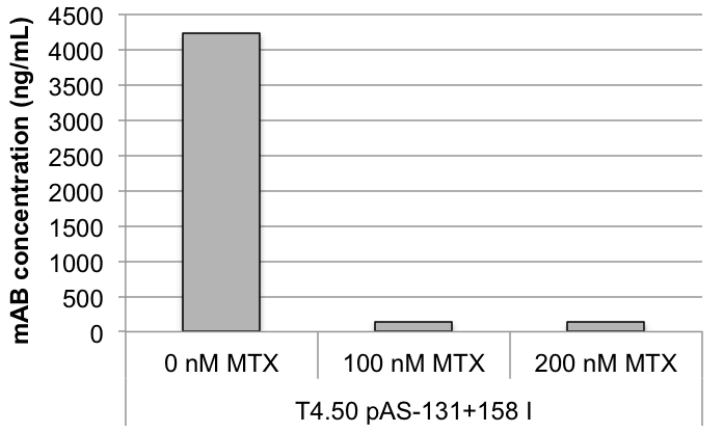

Figure 54: Productivity of stable cell line after MTX selection

The stable CHO-S cell line T4.50 pAS-131+158 I was treated with 100 or 200 nM MTX after the initial P5CS/zeocin selection. Secreted antibody was measured by an IgG/kappa-specific ELISA on day 105 after transfection.

The fluorescent protein expression of the coexpressed RFP and GFP was changed by the MTX treatment, as shown in Figure 55. The untreated stable cell line T4.50 pAS-131+158 I had the lowest RFP expression intensity, but the highest number of double positive cells. Treatment with MTX shifts the cells to a higher expression of RFP, which is encoded on the same vector as the light chain and DHFR. In addition, the MTX treatment reduced the number of GFP-expressing cells in this vector setting, which encodes GFP on the same vector as the heavy chain.

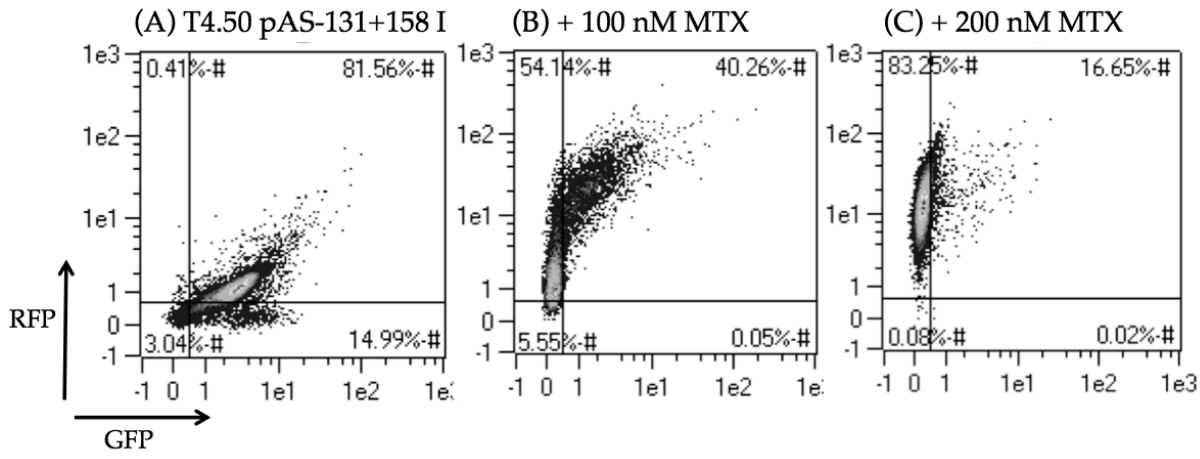

Figure 55: Flow cytometry analysis of MTX treated stable cell lines

The stable CHO-S cell line (A) T4.50 pAS-131+158 I was treated after the initial P5CS/zeocin selection with (B) 100 nM or (C) 200 nM MTX. Coexpressed RFP and GFP were monitored by flow cytometry on day 98 after transfection.

Among the cells transfected with the vectors pAS-131 and pAS-158 (T4.50 and T5.52) and directly selected with MTX only few stable cell lines recovered although different concentrations of MTX and zeocin were tested: All selection media were proline-free and contained different zeocin concentrations (0, 50, 100 ng/mL) combined with different MTX concentrations (50, 100, 200 nM). The cell line generation for each medium was repeated at least in duplicates. The few stable cell lines that did grow up after 3 to 4 month showed massively reduced growth rates making expansion impossible.

These results demonstrate that a combination of P5CS and zeocin selection with a DHFR amplification has to be further optimized.

4 Discussion

Mammalian expression systems are necessary for the production of complex proteins due to their ability of post-translational modifications. These post-translational modifications, such as glycosylation, are often crucial for the biological activity and stability (Hung et al, 2010). Chinese hamster ovary cell lines are the most commonly used hosts for recombinant protein production in mammalian cells, both, for laboratory and biopharmaceutical production. They combine several properties essential for a production cell line including high-level productivity, stability over time, growth in high cell densities, ease of adaptation to serum-free media and suspension, scalability to large-scale production and glycosylation with high similarity to human glycan patterns (Costa et al, 2009). Recombinant proteins produced by CHO cells have been proven to be safe for the use in humans. Several therapeutic proteins have been FDA-approved (Carroll and Al-Rubeai, 2004).

Currently, selection systems used either depend on antibiotic or metabolic selection. Commonly used metabolic selection systems include the DHFR and the GS selection system. Antibiotics have highly toxic effects on cells lacking the corresponding resistance gene (Schiedner et al, 2008). Protein synthesis is inhibited by the aminoglycoside antibiotics hygromycin B and Geneticin (G418, neomycin) due to the induction of misreading of aminoacetyl-tRNA by distorting the ribosomal A site and the translocation process (Cabanas et al, 1978; Vicens and Westhof, 2003). The aminonucleoside antibiotics puromycin and Blasticidin S (Tunkel and Studzinski, 1981; Kuwano et al, 1979) also inhibit protein synthesis but based on the integration into the ribosomal RNA and blockage of further elongation of the rRNA chain (Tunkel and Studzinski, 1981). Regarding the glycopeptide antibiotic zeocin, which belongs to the bleomycins, the exact mode of action is not known. But zeocin is believed to intercalate into DNA and thereby cleave DNA (Ranft et al, 2009; Drocourt et al, 1990). The use of toxic antibiotics has several negative effects: First, these cytotoxic substances do not only inhibit growth of untransfected cells, they also influence cells carrying a resistance gene. As seen in this study addition of zeocin reduced the maximal viable cell density in a fed-batch process. Rodolosse et al (1998) describe that hygromycin B-selected stable cell lines exhibit dramatically changed metabolic properties with strongly increased glucose consumption and lactic acid production. In addition, the frequency of sister chromosome exchange, which is correlated with mutagenic and carcinogenic events, is increased by both aminoglycoside antibiotics, hygromycin B and G418, in a dose-dependent manner in different cell lines transfected with the corresponding resistance genes (McDaniel and Schultz, 1993).

Toxic substances are also used in metabolic selection systems. The DHFR selection system is based on the use of DHFR deficient CHO DG44 cells, therefore not requiring the use of an inhibitor during the initial selection. To archive high-producing cell lines, the DHFR

inhibitor MTX, which acts as a folic acid antagonist, can be used for amplification (Wurm, 2004), but implicates a high cytotoxicity, mutagenicity and teratogenicity (Kaufman et al, 1985; Khera, 1976). The high cytotoxicity can also be observed in humans. Alarcón et al (1989) report major life-threatening, and minor toxic events after MTX treatment in 54% of all patients. In addition, amplified CHO cell lines show heterogenicity in the chromosomal location and the copy number of the amplified sequence. Rearrangements, highly extended regions, joined chromosomes at amplified regions and even circular chromosomes mainly consisting of amplified regions lead to a high genetic instability of the production cell lines (Schiedner et al, 2008; Kim et al, 1998 A).

CHO-K1 cells used for the GS selection are not deficient for the enzyme glutamine synthetase. Since a glutamine-free medium only provides a weak selection pressure, this system requires the permanent use of the cytotoxic GS inhibitor MSX to ensure sufficient selection pressure and prevent deletion or silencing of the foreign DNA (Shaw et al, 1999; Chartrain and Chu, 2008). Recently GS-knockout cell lines generated by zinc-finger-nucleases have been described (Liu et al, 2010). The lack of endogenous GS increased selection stringency and lead to an increased recombinant protein production. But also for the GS-deficient CHO cell line cytotoxic MSX is used in the selection process (Fan et al, 2012). Antibiotics as well as other inhibitory substances need to be removed from the culture medium for the production process of therapeutic proteins to minimize cellular stress and to eliminate the risk of contamination with cytotoxic substances (Kaufman et al, 2008).

One aim of this project was the development of a novel selection system based on the proline metabolism. The goal was to develop a selection system without the need of an inhibitory, toxic substance facilitating fast, reliable and stable generation of producer cell lines expressing suitable amounts of recombinant proteins.

4.1 Characterization of proline metabolism in CHO cells

The CHO cell line is known to be proline auxotroph (Kao and Puck, 1967) due to a deficiency in both available proline catabolic pathways (Baich, 1977; Smith et al, 1980). The ability of spontaneous reversion from a proline auxotroph to a prototroph phenotype has been observed (Kao and Puck, 1967).

The first set of experiments in this study was dedicated to the characterization of the proline metabolism of CHO cells. Hence, parental CHO cell lines (Pro-) were seeded in proline-free medium and after about 10 days single proline prototroph colonies (Pro+) started growing up. Isolated CHO-K1 and CHO DG44 Pro+ cell lines showed similar growth properties in proline-free medium as parental Pro- CHO cell lines in proline-supplemented medium. All media were supplemented with 10% dialyzed FBS containing traces of proline. In contrast, CHO-S Pro+ cell lines showed significantly reduced growth in proline-free medium (data not shown) indicating that the grade of deficiency in proline metabolism differs between

different CHO cell lines. Similar to the results of Kao and Puck (1967), it was found in this study that there are also slight variations between individual Pro+ cell lines isolated from the same parental cell line under identical conditions regarding growth rates. The reversion rate of CHO-K1 from a proline auxotroph to a prototroph phenotype was calculated to be 0.01% to 0.015%. This reversion rate is higher than the one described by Kao and Puck (1967). The used CHO cell line in their studies showed lower reversion rates of $1x10^{-6}$ with reduced plating efficiencies and growth rates of the proline prototroph cell lines in the absence of proline when compared to naturally proline prototroph Chinese hamster lung (CHL) cells. This reduced growth rate is comparable to the CHO-S Pro+ cell lines isolated in this study, but could not be observed in CHO-K1 and CHO DG44 Pro+ cells, indicating that certain CHO cell lines might be more suitable for a proline-based selection system. Reversions to a selection independent phenotype are known for other metabolic selection systems as well. For example, reversion rates from a DHFR-sensitive to a DHFR-insensitive phenotype for the CHO DXB11 have been described between 0.001% (Urlaub and Chasin, 1980) and 0.3% in serum-free suspension media (Sinacore et al, 1996). For CHO DG44, a cell line with deletion of both DHFR alleles, MTX-resistance due to a mutated MTX transport system has been described (Assaraf and Schimke, 1987).

To further characterize the isolated CHO Pro+ cell lines, P5CS and OAT protein levels were analyzed by Western Blot experiments. It was found that OAT expression could not be observed in any parental or Pro+ cell line. P5CS expression could not be detected in parental CHO cells, but was detected in CHO-K1 and CHO DG44 Pro+ cell lines, explaining their ability to grow normally in proline-free medium. This indicates that P5CS and not OAT is the more important pathway for proline synthesis in CHO cells, which correlates with data from plants (Verbruggen and Hermans, 2008) and humans (Krishnan et al, 2008). Comparing the different CHO cell lines, no P5CS expression could be observed in the isolated CHO-S Pro+ cell line, making the CHO-S cell line a promising candidate for a production cell line, which can be selected by P5CS. Further this explains the reduced growth rates of CHO-S Pro+ cell lines in proline-free medium compared to CHO-K1 and CHO DG44 Pro+ cell lines. Sequence analysis of P5CS from Pro- and Pro+ CHO-K1 cell lines revealed no differences in cDNA sequences indicating that the P5CS deficiency of the parental cell line is not due to a mutation in the P5CS DNA sequence as supposed by Kao and Puck (1967) but indicating an up-regulation of P5CS expression during the reversion from a proline auxotroph to a prototroph phenotype, which could be due to an increased transcription rate or increased mRNA stability. This theory is supported by real-time PCR analysis data, confirming different levels of P5CS mRNA in the Pro- and Pro+ CHO-K1 and CHO-S cell lines. The rate of mRNA up-regulation in Pro+ cells shows no direct correlation with the observed P5CS protein levels. These differences between proteomic and genomic analysis have been reported by various groups (Jansen et al, 2002; Pradet-Balade et al, 2001). They are caused by the fact that mRNA levels and resulting protein levels are not only determined by

transcription and translation rates but also by additional control factors. These factors include mRNA stability, export and protein degradation (Pradet-Balade et al, 2001).

4.2 P5CS as novel selection marker for the expression of recombinant proteins

There are two available catabolic pathways for proline synthesis in CHO cells. Both enzymes have been previously used to functionally complement proline auxotroph CHO cells (Brody et al, 1992; Hu et al, 1999). However, no selection system based on the proline metabolism has been described. Several factors were taken into account when deciding which of the key metabolic enzymes to use as a selection marker: First, several publications indicate that the metabolic pathway from glutamate is the more important one under stress conditions. Proline is known to protect plant as well as mammalian cells from stress for example it can act as reactive oxygen species (ROS) scavenger. ROS might be produced by salt and drought stress in plants (Verbruggen and Hermans, 2008) or UVA and osmotic stress in mammalians (Wondrak et al, 2005; Krishnan et al, 2008). It has also been proposed that proline functions as a chaperone, buffers cytosolic pH and balances the cell´s redox status (Verbruggen and Hermans, 2008). In plants, proline is mainly made from ornithine under normal conditions. Under osmotic or drought stress, the glutamate pathway becomes dominant (Kishor et al, 1995; Verbruggen and Hermans, 2008) with the enzyme P5CS as the rate-limiting step of the proline synthesis (Verbruggen and Hermans, 2008). Similarly, Krishnan et al (2008) found that OAT expression is down-regulated during oxygen stress in HEK293, HeLa and HepG2 cells. Since OAT is a bidirectional enzyme, it catalyzes the reaction from ornithine to P5C and back. Under stress conditions, OAT might be down-regulated to avoid any formation of ornithine and instead ensure maximal formation of proline by up-regulation of the P5CS pathway (Krishnan et al, 2008). These findings indicate that under production conditions, which do lead to oxygen stress (Selvarasu et al, 2012; Han et al, 2011) resulting in growth limitations and apoptosis, P5CS might be the more interesting candidate as a selection marker. Second, although Smith and Phang (1979) described CHO cell lines isolated from proline-free medium that only expressed OAT and no P5CS, it was only possible to generate Pro+ CHO cell lines that expressed P5CS and no detectable OAT in this study. This might indicate that the metabolism from glutamate is be predominant in the here used cells and under the given culture conditions. Third, OAT is a bidirectional enzyme catalyzing the proline synthesis as well as the degradation towards ornithine and arginine, resulting in more complex regulation mechanisms (Brody et al, 1992). Therefore, it was decided to test murine P5CS in the P5CS.long splice form, which is not inhibited by ornithine (Hu et al, 1999), as a selection marker.

For expression studies, the enhanced GFP was used as GOI due to its ease of detection via microscopy and flow cytometry. To validate the newly constructed dicistronic expression vector pMACS-CHO, stable cell lines were generated using a standard neomycin antibiotic selection and were then compared to cell lines generated with a two-promoter expression

vector. Consistent with the results of Lucas et al (1996), Pu et al (1998) and Ho et al (2012) coupling of GOI and selection marker by an IRES element resulted in higher producer rates and higher GOI expression levels.

In the next step, it was tried to use the enzyme P5CS as selection marker. Again, GFP was used as a model protein. Already in first experiment it was possible to generate stable CHO-K1 cell lines selected by P5CS in proline-free medium with GFP producer rates of up to 99% However, the respective selection times of over 100 days were too time consuming. Furthermore, these high producer rates could only be obtained in about 50% of all transfected and P5CS-selected cell lines indicating that untransfected cells spontaneously reversed to a Pro+ phenotype and were able to overgrow transfected cells.

Three changes strongly improved the P5CS selection system: First, switching to CHO-S cells as a host cell line; second, optimizing the selection protocol with a harsh split 24 hours after selection and third, using a serum-reduced proline-free medium containing only 5% dialyzed FBS. By these changes, it was possible to generate a fast and reproducible selection system yielding stable cell lines with GFP producer rates of over 90% in less than 16 days. As described previously, CHO-S cells have a lower tendency to reverse to a proline prototroph phenotype and corresponding Pro+ cells express much lower amounts of P5CS compared to CHO-K1 Pro+ cells resulting in slower growth rates in proline-free medium. CHO-S proline prototroph cells spontaneously formed during the selection process in proline-free medium therefore have a growth disadvantage and are more frequently overgrown by cells with stable integration of the expression vector. In contrast, the CHO-K1 Pro+ cells spontaneously formed during selection are able to overgrow transfected cells with stable integration of the expression vector into the genome, because these Pro+ cells express sufficiently high amounts of P5CS to allow normal growth rates. Apart from the cell line used, another crucial factor for the P5CS selection system was found to be the amount of dialyzed FBS in the selection medium. The used lot of dialyzed FBS contained $3.9x10^{-4}$ M proline. Using a medium supplemented with 10% of the dialyzed FBS resulted in a proline concentration of $3.9x10^{-5}$ M. In comparison, Kao and Puck (1967) determined proline requirements of CHO cells to be $3x10^{-5}$ M for a normal plating efficiency and colony size in plating tests with different proline concentrations. The medium Kao and Puck (1967) used was supplemented with 2% non dialyzed FBS and 1.5g/L of the macromolecular fraction of BSA, which both presumably contained traces of proline, therefore the actual proline concentrations might have been higher. A reduction of the supplementation with dialyzed FBS in the selection medium from 10% to 5% reduced proline concentration to $1.95x10^{-5}$ M, a level at which no normal colony formation was observed in studies of Kao and Puck (1967).

To validate the P5CS selection system for the expression of secreted recombinant proteins, the cytokine human transforming growth factor beta 1 (hTGF-β1) was chosen as one model protein. Human TGF-β1 is a homodimeric protein interacting with the latency associated

protein (LAP) in its latent form, which is encoded in the N-terminal region of the hTGF-β1 gene and is cleaved of during maturation. This cytokine plays an important role in immunosuppression and immunotolerance, development and differentiation as well as mutagenesis (Beatson et al, 2011). To determine secreted hTGF-β1 concentrations in the cell culture supernatant, the latent form bound to LAP had to be activated by acid: Hydrochloric acid was thus added to activate the hTGF-β1. The activation was followed by a neutralization with sodium hydroxide to ensure a pH range suitable for binding to an ELISA plate. Practical application revealed one challenge: In particular, samples were wither not acidified enough leading to incomplete activation or the neutralization was not accurate enough leading to a pH not optimal for binding of the samples to the ELISA plates. Both factors led to high variations between doublet samples. Using commercially available standard acid and base solutions, it was possible to reduce problems of variations between sample doublets and ensure a suitable pH after neutralization. However, since there does not exist a latent hTGF-β1 standard as an activation control, it could not be guaranteed that the activation of latent samples was 100% efficient, which might have led to an underestimation of secreted hTGF-β1 concentrations.

Since hTGF-β1 is a homodimer encoded by only one gene, it is a suitable model protein to directly compare different selection systems in a one-vector strategy. Therefore, the novel P5CS selection system was compared with three antibiotic selection systems. Expression levels of all tested stable CHO-S cell lines selected by P5CS or by antibiotic selection (neomycin, hygromycin B or zeocin selection) confirmed the fast selection time observed expressing GFP. Less than 20 days after transfection, cell lines already secreted hTGF-β1 at levels, which remained stable over the following 30 days of culture. The novel P5CS selection system led to secreted hTGF-β1 amounts comparable to a conventional neomycin or hygromycin B selection. Zeocin selection enabled hTGF-β1 concentrations in the supernatant about three- to four-fold higher. Unfortunately, there are not many publications available, which compare the efficiency of different selection markers in CHO or other mammalian cells. Therefore, there are no results of other antibiotic selection systems to compare the superior expression of zeocin-selected cells to. Using the novel P5CS selection system hTGF-β1 concentrations comparable to antibiotic selection systems routinely used to select stably transfected cells for research applications could be obtained. Thus, the P5CS selection system might be a promising alternative due to the major advantages of being a metabolic system without the need for any cytotoxic substances. This might also be of special interest for the modification of parental CHO cell lines used for recombinant protein production. In contrast to the selection systems for the recombinant protein expression requiring amplifiable system with extremely high yields, overexpressing genes to modify the cell line requires only a moderate level of expression. Targets for engineering might include anti-apoptosis factors such as Bcl-2, Bcl-xL or Aven or metabolic enzymes such as pyruvate carboxylase or lactate

dehydrogenase to avoid lactate accumulation (Costa et al, 2009). Other host cell modifications are based on the ER secretory pathway by overexpressing chaperons, protein disulphide isomerase or the X-box binding protein 1 as a regulator of many critical genes of the secretory pathway (Costa et al, 2009). Also modification of the glycosylation pattern might make knock-in cell lines necessary. For example overexpression of galactosyltransferase, sialyltransferase and N-acetylglucosaminyltransferase have been reported (Costa et al, 2009). Selection of cells overexpressing these proteins is usually antibiotic-based. Neomycin (Mastrangelo et al, 2000; Sauerwald et al, 2002), puromycin (Figueroa et al, 2007; Figueroa et al, 2004) and hygromycin B (Jung et al, 2002; Sauerwald et al, 2002; Mohan et al, 2007) have been reported to be used as selection markers. As described above, antibiotic selection has negative effects on cell growth, even in cells expressing resistance genes (Rodolosse et al, 1998; McDaniel and Schultz, 1993). In addition to the selection marker needed to modify the parental cell line, further selection markers for the expression of the recombinant target protein have to be used, adding up to an increased toxic burden for the cells. Using the enzyme P5CS as a selection marker, which leads to similar expression levels as mentioned antibiotic systems, would only require the use of a proline-free medium to generate and maintain the parental cell line. Thereby, the modification would not bring any additional cytotoxic burden. In addition the enzyme P5CS has been reported to be connected to oxygen stress response. P5CS expression is increased in plants (Kishor et al, 1995; Verbruggen and Hermans, 2008) as well as human cells (Krishnan et al, 2008) challenged with oxygen stress by direct treatment with H_2O_2 and in plants drought or salt stress leading to formation of reactive oxygen species (Verbruggen and Hermans, 2008). Recombinant overexpression of P5CS can rescue plant and mammalian cells from oxygen stress (Kishor et al, 1995; Krishnan et al, 2008). Reactive oxygen species are known to induce apoptosis (Yun et al, 2003 A). Generation of reactive oxygen species during the production process represents a potential inducer of cell death (Laken and Leonard, 2001) since apoptosis can account for a major part of cell death during a production (Majors et al, 2007). Addition of insulin, glutathione and iron chelators have been proven to reduce intracellular levels of reactive oxygen species and enhance cell survival and productivity (Yun et al, 2003 A; Yun et al, 2003 B). A modified cell line overexpressing P5CS as a selection marker thereby might bring the additional advantage of rescuing cells from apoptosis.

In addition to the expression studies with the cytokine hTGF-β1, three different monoclonal antibodies were produced as model proteins using the novel P5CS selection system: a human anti-CD303 antibody with humanized variable regions and chimeric anti-Biotin and anti-CD14 antibodies with murine variable regions. All antibodies contained human constant IgG1 and kappa regions. Expressing recombinant proteins with two or more subunits gives a choice of different vector designs. Both subunits can either be expressed on two different expression vectors selected by different markers (two-vector strategy) (Pichler et al, 2011;

Sleiman et al, 2008; Brezinsky et al, 2003) or on one single expression vector (one-vector strategy) by different promoters (dual-gene vector) (Schlatter et al, 2005; Hung et al, 2010) or in a dicistronic construct coupled by IRES elements (Li et al, 2007), 2A peptides (Fang et al, 2005), alternative splicing (Fallot et al, 2009) or intein-mediated protein splicing (Kunes et al, 2009). Although a one-vector strategy brings the advantage of correlating expression levels (Li et al, 2007) especially when using coupled expression of the two GOIs again, it was decided two use a two-vector strategy in this study. As described above, the generated pMACS-CHO expression vector already contains a dicistronic cassette with coupled expression of GOI and selection marker to facilitate an increase in producer rates. To ensure coupling of GOI and selection marker expression, the expression of both antibody chains on one vector would create the need for a tricistronic construct. But all of the aforementioned coupling elements have some major drawbacks for coupling of two recombinant protein subunits. Using 2A elements, equimolar expression of first and second gene is caused, which is not wanted for all recombinant proteins. For example, some publications describe that for the expression of antibodies an excess of light chain leads to an increased production (Bibila and Flickinger 1991; Schlatter et al, 2005). In addition, after the "cleavage" at the 2A element, the 2A residue amino acids remain on both chains, which might be unwanted for therapeutic applications. Using an IRES element, it is possible to vary heavy and light chain expression (Li et al, 2007), but the efficiency of the IRES element is dependent on the surrounding sequences (Davies and Kaufman, 1992). Therefore, optimization might be necessary for different antibody constructs. Splicing-based vectors have only been described in few publications for antibody expression and do not seem to be commonly used. In addition to the mentioned problems with a one-vector strategy, using a two-vector strategy brings the advantage to balance heavy and light chain expression levels or other proteins´ subunit expression via the used selection marker combination. To identify such an optimal combination of P5CS with another selection marker, the anti-CD303 antibody was expressed using different combination of P5CS, GS and antibiotic selection markers. The highest secreted antibody concentrations were reached with a combination of heavy chain selection by P5CS and light chain selection by zeocin. This correlates to the result of the hTGF-β1 expression in this study; at which zeocin was the stronger selection marker, and also with several publications indicating that a higher light chain expression might be advantageous (Bibila and Flickinger, 1991; Schlatter et al, 2005). To compare the P5CS selection system to an antibiotic system again, it was decided to keep the zeocin selection for the light chain expression and replace the P5CS by neomycin selection for the heavy chain expression. Stable producer cell lines expressing the three different antibodies revealed for all tested constructs that the combination of P5CS and zeocin selection led to similar antibody concentrations as the neomycin and zeocin selection. Expressing the anti-Biotin and anti-CD14 antibodies, the use of neomycin and zeocin selection resulted in slightly higher antibody productivity regarding the stable cell lines as well as the clone distribution after

limiting dilution. In contrast, expressing the humanized anti-CD303, the P5CS and zeocin selection led to higher antibody concentrations. In general, expressing the chimeric antibodies consisting of murine variable regions and human constant regions led to significantly lower antibody concentrations compared to the humanized antibody. Both, intracellular staining of the heavy and light chains as well as the clone distribution after limiting dilution revealed low numbers of non-producing cells, for all tested antibody constructs and selection marker combinations. Double producer rates, defined as cells expressing heavy and light chain intracellularly or secreting complete antibody, were determined to be higher than 75% proving the potency of the dicistronic vector design.

Isolated clones expressing the anti-CD303 antibody and selected by the novel selection system P5CS in combination with zeocin were used for expression studies in shake flasks in batch and fed-batch processes. A batch process is the most simple and therefore most reliable process for clone screening. The shake flask or bioreactor is initially inoculated with cells in appropriate production medium and cells are allowed to grow to determined cell numbers and viabilities until the supernatant is harvested. During culture nutrients decrease and toxic waste increases limiting the maximum cell density (Rodrigues et al, 2010). In a fed-batch process, the culture is fed with concentrated feed supplements continuously or once daily. This reduces nutrient limitations and thereby enables higher cell densities and prolongs culture time. Thereby higher final product concentrations are enabled. Although accumulation of growth-inhibitory waste cannot be avoided, fed-batch processes are still a common production mode due to their scalability, ease of operation and increased product yields compared to batch processes (Rodrigues et al, 2010).

In a batch process, two of the ten analyzed clones expressed more than 50 mg/L recombinant antibody with specific productivities of up to 10 pcd in the proline-free production medium CHO MACS CD, which contained zeocin concentrations as also used for the selection process. To find a more suitable production medium, the two highest producing clones were adapted to five different commercially available production media all containing proline. This led to a cultivation time of about three to four weeks in the presence of proline. The following batch production was carried out in the presence of proline and without zeocin. Analysis of the antibody concentrations revealed about three-fold reduced productivities compared to the previous batch process with selection pressure indicating instability of the clones, although it cannot be said whether the titer decrease was mainly due to an instability of the P5CS or zeocin selection systems or a combination of both. These observations about instability of clones in medium without selection pressure will be discussed later. The previously used CHO MACS CD, in this batch process supplemented with proline, facilitated growth in highest viable cell densities with antibody concentrations similar to those of the HyClone SFM4CHO medium, which led to highest antibody concentrations in this experiment.

A high stability of production clones is a desirable goal of cell line development. Upscaling cells for a 1,000 L bioreactor requires about 50 to 60 cell generations (Dorai et al, 2012; Bailey et al, 2012) and necessitates a stable productivity over at least this time frame to ensure predictable and constantly high recombinant protein yields (Wurm, 2004). In addition, clonal instability may compromise regulatory approval of proteins manufactured for therapeutic applications (Kim et al, 2011). Loss of productivity may occur rapidly or gradually over several passages in absence but also in presence of selection pressure (Kim et al, 2011; Jun et al, 2006) and has been reported in CHO cells both for the DHFR (Chusainow et al, 2008; Kim et al, 1998 A; Kim et al, 1998 B) and the GS selection system (Jun et al, 2006; Kim et al, 2011; Bailey et al, 2012; Dorai et al, 2012). According to Kim et al (2011) and Dorai et al (2012), clonal instability is a widespread problem affecting 40 to 60% of all production cell lines, in which instability is defined as a 30% decline in productivity over a period of 60 generations (Dorai et al, 2012; Bailey et al, 2012). The molecular mechanisms that cause instability are diverse and range from loss of transgene copies (Kim et al, 1998 A; Kim et al, 2011) to a decline in recombinant gene transcription with gene silencing. This is mainly due to promoter methylation (Chusainow et al, 2008; Kim et al, 2011) and leads to selection system- but also clone-dependent instabilities (Kim et al, 2011).

The fed-batch production in this study was carried out in CHO MACS CD initially without proline. From day 3 on daily feeds supplemented the culture medium with proline. Different zeocin concentrations revealed that both tested clones showed a reduction in the maximal reached titer of 5 to 12 % when cultivated in halved zeocin concentration and 25 % to 54 % respectively when cultivated without zeocin. The influence of the proline supplementation due to the daily feed with CHO MACS Feed Supplement could not be addressed in this experiment. Batch experiments with different media containing proline (for over 4 weeks) and no zeocin (only for the batch process) revealed a reduction in final antibody concentration by 75%. Again both selection markers might have caused this instability. To specifically analyze the stability of the P5CS selection system, CHO-S clones expressing hTGF-β1 were used. The clones were selected by only P5CS in a one-vector strategy and isolated by FACS using the coexpressed GFP. For stability tests clones were cultivated in parallel in media with and without proline. After 17 days in culture, a time frame that would be sufficient for a usual batch or fed-batch production, no significant titer reduction could be observed. In contrast, all clones showed productivities reduced by up to 42% decrease after 40 days without selection pressure. These results combined indicate that the addition of proline due to a feed in a fed-batch process would probably not have a negative effect on the productivity since the P5CS selection system seemed to be stable in all tested hTGF-β1-expressing clones for this time period. Since no cytotoxic substances are needed for the P5CS selection system, removal of selection pressure for the production process only would be practical. The removal or even reduction of zeocin selection pressure immediately led to a decrease in productivity indicating that the analyzed clones might be more instable for the

zeocin selection than for the P5CS selection. The massive maximal titer decrease observed during the batch production in different media fits into this picture. Clones were cultivated for over 4 weeks in proline-containing media and the zeocin was removed during the batch process, which might have led to an added decrease in productivity. Although instabilities were tested with different systems (antibody in a two-vector system and cytokine in a one-vector system), instabilities of both selection systems caused productivity decrease in the range of added decreases for both rapid zeocin and delayed P5CS instability. To further characterize the P5CS selection system, a comparison of the stability of different selection systems to each other directly in a one-vector system to obtain more reliable results would be an interesting aspect.

In the fed-batch process, antibody concentrations of over 140 mg/L could be reached for both tested clones. This equates to an increase from batch to fed-batch process of 2.3- to 2.5-fold. Maximally reached viable cell densities could also be massively improved by the daily feeds. Compared to the batch process the increases range between 3.8- and 3.9-fold with 2-fold prolonged culture times. Both antibody titers as well as viable cell numbers increases were remarkably comparable between the tested clones. Several publications compare batch and fed-batch processes with differing results. The final product concentration can be enhanced in all studies, but increases regarding viable cell concentration and culture prolongation differs. Han et al (2011) producing antibody in CHO cells in a 2 L bioreactor with oxygen and pH control and daily feeds during the fed-batch and Altamirano et al (2004) expressing tPA in CHO observed no increase of cell number, but doubled culture time and an antibody concentration increase of about 1.5-fold in the fed-batch process. In contrast, Jardon et al (2012) also producing tPA in CHO cells in shake flasks with manual pH control saw an increase in culture time (about 2-fold), product concentration (about 4-fold) and also cell concentration (1.8-fold) using a high-feed protocol. Invitrogen reports an antibody titer increase of about 6-fold for the CD OptiCHO system with highest feed concentrations, but only low viable cell density increase and low culture time elongation[5]. Comparing these improvements from batch to fed-batch with the results from this study reveals that the observed product concentration increase and culture time prolongation is in the range of reported enhancement. However, the nearly 4-fold increase in viable cell density, which is higher than in any of the mentioned comparisons of batch and fed-batch, was unexpected.
The maximal antibody concentration reached using the P5CS and zeocin selection for antibody expression in a first fed-batch process was 140 mg/L for two individual clones isolated by limiting dilution with screening of a low amount of clones. These productivities

[5] http://www.invitrogen.com/etc/medialib/en/filelibrary/bioproduction/pdfs.Par.7260.File.dat/CHO%20CD%20EfficientFeed%20Product%20Flyer.pdf, 25.10.2012

are much lower than reported titers for amplified systems. For the GS system[6] as well as for the DHFR system (Gagnon et al, 2011; Schulz et al, 2010) antibody concentrations produced by stable CHO clones in improved production processes of over 8 g/L have been reported. But these maximal titers are obtained from clones manufactured for therapeutic antibody production, implying the need of high productivities in order to reduce costs during the production process. These high-producing clones are generated by optimized selection systems and, in addition, by optimized high-throughput clone isolation. Production processes in bioreactors are optimized specifically for each clone (Gagnon et al, 2011; Schulz et al, 2010). As an example for this laborious and time-consuming effort to push clones to these productivities, Gagnon et al (2011) described a pH-controlled delivery of glucose during fed-batch bioreactor productions enabling a DHFR-selected clone previously producing 0.9 g/L to reach titers of 5.4 g/L. In comparison, Lee and Lee (2012) also used amplified DHFR-selected clones in a standard batch process and obtained significantly lower antibody titers of only 600 mg/L emphasizing the importance of an optimized production process. Not only the standard production process used is a factor that has to be taken into account for the lower titers observed in this study. The P5CS system is a non-amplified selection system. The amplification during DHFR selection using MTX can lead to a 6-fold titer increase as observed by Surabattula et al (2011) and 10-fold increase in specific productivities as observed by Lipscomb et al (2005). Agrawal and Bal (2012) described an up to 200-fold increase in GS copy numbers after MSX amplification. In general, gene amplification of GOI and selection marker should correlate as well as the amplification and expression level, although there are limitations at high amplification rates (Chusainow et al, 2009).

Usually, DHFR-deficient CHO strains are used for a DHFR amplification (Kim et al, 1998 A and B). Recently, Invitrogen described the use of CHO-S cells in combination with a DHFR amplification applying a double selection with puromycin and MTX directly after transfection[7]. Similar to this Invitrogen protocol, the DHFR gene controlled by a SV40 promoter was integrated into a pMACS-CHO II vector encoding the anti-CD303 light chain, a zeocin resistance protein and RFP. This novel pAS-158 vector was cotransfected into CHO-S cells with a P5CS-selected heavy chain–vector (pAS-131). Although using similar concentrations of MTX as proposed by Invitrogen and testing different selection strategies (P5CS, zeocin and MTX selection; P5CS and MTX selection; zeocin and P5CS selection followed by MTX amplification), only few cells were able to recover to stable cell lines. These stable cell lines had lower productivities than MTX-untreated cell lines. Flow cytometry analysis indicated that the RFP, which was encoded on the light chain vector, was amplified, but GFP expression, which was encoded on the heavy chain vector, was lost. During the long

[6] http://www.lonza.com/custom-manufacturing/development-technologies/gs-gene-expression-system/advantages-of-the-gs-system.aspx, 25.10.2012
[7] http://products.invitrogen.com/ivgn/product/A1369601?ICID=search-product, 25.10.2012

recovery time, the P5CS selection might have been too weak to avoid loss of GFP and heavy chain expression. To improve these first approaches with a combination of P5CS and DHFR selection, a first step must be the improvement of the selection protocol to reduce the time needed. In addition, it might be favorable to test different vector settings: with the P5CS and DHFR on one vector and zeocin resistance gene on the other; with DHFR on both the P5CS- and zeocin-selected vector or even on one vector. In such a one-vector strategy a dual-promoter strategy might be promising to keep the coupling of GOI and selection marker, which has been proven to be very successful in this study.

In all probability, an optimized process could increase the currently reached titers with a combination of P5CS and zeocin selection, but the focus of this project was a novel expression system for cell line development. For the purpose of validating the generated clones the used process was suitable. To further enhance the productivity of the P5CS selection system, the cell line development still offers room for improvement: First, as described, a combination of P5CS selection with an amplifiable system such as DHFR might be favorable to establish a fast system for medium scale productivities and, if necessary, clones might be further optimized by amplification. Second, a P5CS- and OAT-knockout cell line might further improve the selection as described for GS-knockout mutants used in the GS selection system (Fan et al, 2012). Third, optimizing the high-producer identification and isolation can greatly enhance productivity. Developing such an enrichment system for FACS as well as MACS was addressed in the second part of this study.

4.3 Development of a novel cell enrichment system

In addition to a suitable selection system, the generation of cell lines with high productivities crucially depends on the isolation of clones with extraordinary high productivities. Limiting dilution as a traditional clone isolation process has the drawback of being very time-consuming. Even automation of this process allows only relatively low numbers of cells to be analyzed (Chartrain and Chu, 2008). Therefore, several techniques have been developed allowing screening of larger cell numbers and isolating only those cells with productivities above average. Identification of high-producing cells is mainly accomplished by two alternatives, the detection of produced recombinant protein or by coexpression of a reporter protein (Carroll and Al-Rubeai, 2004). Currently, high-producer isolation systems mainly focus on FACS for clone isolation and only few enrichment systems based on magnetic separation have been described. Carroll and Al-Rubeai (2005) describe a MACS-based high-producer enrichment protocol using affinity capture surface display (ACSD). Hoch (2010) established an "Antibody CHO Capture Assay" detecting the secreted recombinant antibodies with a capture antibody in combination with MACS enrichment. The second aim of this study was thus the establishment of an enrichment process for high-producers that can be used for FACS and also MACS and is suitable to be used in combination with the P5CS selection system. Instead of detecting the secreted antibody as tested by Hoch (2012)

and Carroll and Al-Rubeai (2005), the approach was based on the coexpression of a fluorescent reporter protein. This had several advantages: First, due to its fluorescence no additional labeling was necessary thereby reducing handling steps and subsequently cell stress during the FACS sort. Second, the fluorescent protein could be targeted to the membrane allowing staining with microbead-coupled antibodies for MACS enrichment. Third, the fluorescent protein might be additionally used to monitor the cell lines production levels and stability by flow cytometry without the need for any additional staining making this an easier and faster monitoring tool than ELISA screens.

The coupled expression of GOI and selection marker has been proven to successfully facilitate high producer rates in the selection process. Therefore, the dicistronic pMACS-CHO expression vector was used as a backbone. The additional reporter protein had to be coupled to the GOI as well to reach correlating expression intensities to make the reporter protein usable to predict expression levels. A 2A peptide sequence was chosen as a coupling element to obtain a tricistronic expression cassette with the additional reporter protein. The expression of IRES-controlled genes is considerably lower when compared to cap-translated genes (Szymczak and Vignali, 2005), which is wanted in case of the selection marker. But a second IRES might lead to expression levels of selection marker or reporter protein depending on the order, which might be to low to survive selection or to be detected. A 2A element leads to about equimolar expression ratios of the gene up- and downstream of the 2A peptide since both genes are encoded as one fusion gene and are separated during translation (Szymczak and Vignali, 2005). Several problems were observed using the first tricistronic vector pMACS-CHO-2A with the order GOI – IRES - selection marker - F2A – reporter protein: Transfected cells did not survive the standard applied selection pressure, and, in addition, expressing a membrane-bound GFP as a reporter protein, it was not possible to stain GFP on the cell surface. Both observations indicated that selection marker and reporter protein were not properly cleaved resulting in a fusion protein that was not active as a selection marker and not localized at the membrane. This problem was solved by the use of a different 2A peptide that had proven to facilitate cleavage (Lock, 2012) with an additional serine-glycine linker allowing more flexibility in confirmation and improving the ribosomal-skip mechanism (Szymczak and Vignali, 2005). In addition, the order of reporter protein and selection marker was switched since stable cell lines with loss of reporter protein had been observed. Having the selection marker at the end of the expression cassette ensured that the whole cassette had to be expressed to gain resistance. With this novel tricistronic expression vector pMACS-CHO II with the order GOI – IRES- reporter protein – T2A – selection marker, it was possible to generate stable cell lines according to the standard protocol. This indicated that cells were able to generate sufficient amounts of functional selection marker. The membrane-bound GFP was detectable in a surface staining also indicating a proper cotranslational cleavage and correct localization. By monitoring GFP and

RFP coexpression of an antibody-producing stable cell line, it could be proven that the developed selection system delivered high producer rates of over 90% in less than 20 days.

Getting the cotranslationally separated proteins to their intended localization might be an issue when using 2A peptides. De Felipe and Ryan (2004) studied the effect of proteins with different localizations coupled by 2A peptide sequences and observed several mislocalizations of the second protein. For example, a setting where only the first protein contained a signal sequence resulted in translocation of both proteins into the ER, presumably due to the second protein "slipstreaming" through the translocon formed by the first protein. But this incorrect localization is dependent on the order and intended localization of proteins. Other studies describe cleavage events resulting in correct localization. For example, expression of two secreted subunits of the cytokine IL-12 resulted in efficient secretion (Chaplin et al, 1999). Tang et al (2009) also used some additional amino acids to improve the cleavage environment and were able to express a nucleus-localized Cre recombinase, a secreted Noggin or two light-activated membrane-bound proteins (Channelrhodopsin2 and Halorhodopsin) at the first position coupled to a second cytosolic fluorescent protein, all resulting in correct distributions. Using the improved tricistronic pMACS-CHO II vector, no further indications for mislocalized proteins expressing a cytosolic or membrane-bound fluorescent protein before the 2A peptide in combination with the cytosolic zeocin resistance protein or the mitochondrial P5CS could be observed in this study.

The influence of a di- or even tricistronic construct on the expression of the GOI is not clearly defined. Chinnasamy et al (2006) compare a monocistronic construct expressing O6-methylguanine-DNA-methyltransferase (MGMT) to two different dicistronic constructs with an IRES element or a 2A peptide. In the dicistronic construct, the MGMT expression was reduced by about 15% to 20%. Studies with the dicistronic pMACS-CHO vector expressing fluorescent proteins as GOI with different genes at the second position behind the IRES element revealed similar reduction rates in transient transfections. In stable transfections, however, the advantage of the coupled expression of GOI and selection marker led to dramatically higher producer rates and about 3-fold higher expression levels compared to a two-promoter vector. For a tricistronic construct consisting of MGMT-2A-HOXB4-IRES-GFP, Chinnasamy et al (2006) reported an even higher reduction of MGMT expression levels of about 80% compared to a monocistronic constructs. Similarly, Osborn et al (2005) showed that expressing iduronidase (IDUA), a lysosomal enzyme involved in glycosaminoglycan (GAG) degradation, in a monocistronic construct or a tricistronic construct with coexpression of luciferase and a fluorescent protein coupled by two different 2A peptides resulted in a nearly 3-fold lower activity of IDUA in the tricistronic constructs. Whereat measuring the activity, it is not clear whether it was reduced due to lower expression levels or due to reduced activity, for example, caused by the additional 2A peptide amino acids. Such a dramatic reduction of GOI expression would not be suitable for an expression vector made

for high-level recombinant protein reduction. One more promising result regarding tricistronic vectors was published by Bouabe et al (2008), who expressed luciferase in a monocistronic construct compared to di- and tricistronic constructs containing one or two additional IRES-GFP blocks. Bouable et al (2008) did not see any effect of these multiple tandem-IRES on the upstream cap-dependent gene expression. Expression experiments with hTGF-β1 selected by P5CS in a dicistronic pMACS-CHO vector or tricistronic pMACS-CHO II vectors coexpressing GFP or the membrane-bound GFP showed usual variations between stable cell lines, but no negative effect of the tricistronic vector of the GOI expression. These results indicate that this vector might be suitable for recombinant protein expression. These inconsistencies in the results of different studies demonstrate that a reduction of GOI expression might strongly depend on the used elements and their combination as well as on whether the expression is transient or stable since the stringency of selection marker expression has an impact on the GOI expression.

Once the applicability of the tricistronic vector pMACS-CHO II was proven, FACS-sorted clones using the coexpressed fluorescent reporter proteins for sorting were directly compared to clones isolated by a traditional limiting dilution. Expressing a humanized antibody, the enrichment regarding the highest producing clone from the starting cell line was 10-fold for the FACS-sorted clones and 5.7-fold for the limiting dilution clones. This resulted in a 1.8-fold higher increase from FACS compared to the limiting dilution. During the initial screen in 96-well plates, even higher titers of FACS isolated clones correlating to an enrichment of 27-fold could be observed. But nearly all clones lost productivity even under selection pressure. As discussed above, instability of production cell lines is a major and very common issue. To get further evidence whether the instability observed in this study was caused by the FACS enrichment process, since such rapid productivity loss under selection pressure has not been seen before in any limiting dilution, the clone isolation process by FACS was repeated with a pre-enriched cell line as well as cell lines expressing hTGF-β1. In none of the following clone isolations by FACS an increased instability of clones could be observed. Repeating the FACS-based clone isolation for an hTGF-β1-expressing, P5CS-selected and GFP-coexpressing cell line could confirm the obtained enrichment rates from the first sort with a 8.8-fold enrichment. These hTGF-β1 clones were also tested in a batch process to ensure the ability of adaptation to serum-free conditions in the shake incubator and stability of productivity. As in a batch process with antibody-producing clones isolated by limiting dilution, viable cell densities of up to $1x10^7$ cells/mL were reached. Compared to the production of recombinant antibodies, maximal titers of up to 0.27 mg/L as well as specific productivities of up to 0.03 pcd were significantly lower. This observation has already been made during stable cell line generation and fits to published hTGF-β1 productivities: Ramani and Kondaiah (1998) report titers of 0.97 mg/L in a non-amplified, neomycin-selected clone. Amplified clones either isolated by DHFR selection (Bourdrel et al,

1993) or GS selection (Zou and Sun, 2004) reach titers between 7 and 30 mg/L.

Not for all enrichment systems described in the introduction, details about their enrichment factors are given. But comparing the stated enrichment factors of other FACS-based enrichment systems to the system developed in this study reveals a similar range. Brezinsky et al (2003) reported an increase in specific productivity of 10-fold expressing recombinant antibodies using a surface fluorescent antibody staining for detection. Lower enrichment factors were reported by Meng et al (2000), who expressed the vascular endothelial growth factor (VEGF) in a vector setting with coupled GOI and selection marker expression and a separate expression unit for a fluorescent marker reaching an enrichment for FACS-sorted clones of 6-fold regarding the specific productivity. Cairns et al (2011) expressed both a soluble Fc fusion protein and a monoclonal antibody coexpressing CD52 and CD59 by a non-AUG initiation codon with selection markers controlled by a separate promoter. Next, Cairns et al (2003) sorted the top 0.5% cells and compared these clones to clones obtained by limiting dilution. The improvement from the starting cell line to the top limiting dilution (1.5- to 2.9-fold) or FACS-sorted clones (2.4- to 3.5-fold) is much lower than the enrichment rates obtained in this study. In contrast, there is one group describing higher enrichment rates. Sleiman et al (2008) were able to improve titers of an antibody-expressing cell line coexpressing fluorescent proteins via IRES elements with separate promoters for the selection markers up to 30-fold from the starting cell line to the top FACS clone.

The increased enrichment rates of the developed FACS-clone isolation system demonstrate the potency and applicability of this method. In a further characterization step, the correlation of GFP fluorescence, secreted hTGF-β1 and intracellular hTGF-β1 of FACS-sorted hTGF-β1-expressing, P5CS-selected and GFP-coexpressing clones were analyzed. A strong correlation between intracellular hTGF-β1 levels and GFP fluorescence (R^2= 0.83), but not between secreted hTGF-β1 levels and GFP fluorescence (R^2= 0.07) due to a missing correlation of intracellular and secreted hTGF-β1 levels (R^2= 0.09) could be observed. The question whether intracellular and secreted protein levels correlate, has been addressed in several studies with differing results. The intracellular amount of recombinant proteins has been found to correlate in some cases with production rates (Borth et al, 2000). For example, Borth et al (1996) and Strutzenberger et al (1999) found that the intracellular light chain levels correlated better to the overall antibody secretion than the heavy chain levels. In contrast, there are some publications that suggest different levels of intracellular and secreted product in stable CHO cell lines and clones supporting the results obtained in this study (Pendse et al, 1992; Pichler et al, 2011).

Publications of different FACS-based clone enrichment systems state the coefficient of determination R^2 as an indication how well high-producing clones can be isolated according to the flow cytometry signal. Since different systems calculate the coefficient of determination from different parameters, a one-to-one comparison is not possible. From the

previously described systems coefficients of determination were R^2= 0.7 in the separate expression unit GFP-based system of Meng et al (2000) or R^2= 0.9 in YFP/-CFP-based system of Sleimann et al (2008) both correlation fluorescence protein intensity and specific productivity. The coefficients of determination in the non-AUG initiation codon based system of Cairnes et al (2011) was R^2= 0.7 correlation fluorescence protein intensity and titer. Other systems with even lower coefficients of determination also enabled high producer enrichment. For example both Freimark et al (2010) using GFP-coexpression to identify high-producers by a plate reader and Kober et al (2012) using a fluorescent ER reporter CHO cell line reported a correlation of fluorescence intensity and recombinant protein titer with a coefficients of determination R^2= 0.6. The system developed in this study with a coefficient of determination R^2= 0.8 correlating fluorescent intensity and intracellular protein levels is in the range of the mentioned publications. To further improve the clone selection with the developed tricistronic pMACS-CHO II expression vector, it might be favorable to use a fluorescent reporter protein that enters the secretory pathway to closer mimic the secreted recombinant proteins and get a better correlation to the final secreted product by the identification of cells with not only high production levels but also high secretion capacities. One strong indicator that this strategy might be very successful is based on the data from antibody-producing clones isolated by two rounds of subcloning by FACS according to their GFP and RFP fluorescence compared to clones obtained first by four MACS-enrichment steps followed by a FACS-cloning according to the membrane-bound GFP and RFP fluorescence wherein the GFP fluorescence was encoded on the light chain vector. Although only one of the antibody chains has been switched from a cytosolic to a membrane-bound fluorescent protein, the amount of antibody secretion in relation to the GFP fluorescence intensity could be increased from a coefficient of determination R^2= 0.0, indicating no correlation, to R^2= 0.5, a coefficient of determination that is comparable to some publications (Freimark et al 2010; Kober et al, 2012) with successful enrichment systems.

Using a membrane-bound fluorescent reporter protein allows the combination of clone isolation by FACS with previous MACS enrichment steps. Using magnetic assisted cell enrichment for high-producer selection, it is important to have an optimized level of reporter protein expression. All cells expressing the reporter protein are labeled with magnetic beads. A too low amount of reporter proteins leads to a magnetic load too low to retain cells on the column. A too high reporter protein amount results in sufficient labeling and but retainment of all cells not leading to any enrichment. Expressing a recombinant antibody using a combination of zeocin selection and the membrane-bound GFP on one vector, the GFP levels were usable for MACS enrichment. Titers of an antibody-expressing cell line could be improved in each MACS step up to 1.7-fold. Using a combination of P5CS and the membrane-bound GFP on one vector to express hTGF-β1, the enrichment protocol had to be optimized. But using a monoclonal antibody for direct labeling, enrichment rates of 10.6-fold could be reached. The direct labeling with the monoclonal antibody led to higher labeling

intensities of cells compared to the polyclonal indirect approach, thereby being more suitable for cells expressing low amounts of surface reporter. For a higher amount of surface molecules, the polyclonal indirect labeling might be favorable to reach the optimal intensity of magnetic labeling. Expressing a recombinant antibody in a two-vector strategy and enriching only one chain already led to enrichment factors of up to 1.7-fold. This method could be improved using a releasable magnetic labeling system (Miltenyi, EP 0819250B1), thereby having the ability to enrich two different surface reporter proteins in a sequential manner.

Carroll and Al-Rubeai (2005) described a MACS-based high-producer enrichment protocol using affinity capture surface display (ACSD). They were able to enrich NS0 cells expressing a recombinant antibody about 4-fold and stated the very rapid and simple protocol as one major advantage in contrast to FACS. Bort et al (2010) using FACS as well as MACS to select a CHO-K1 cell line growing in glutamine-free medium found that both sorting systems gave similar results and confirmed the MACS protocol being faster and easier to handle. The FACS protocol brings the advantage of being able to set cut-offs and to finer discriminate between healthy and dead cells (Bort et al, 2010), although dead cells have never been an issue during MACS enrichment in this study if a starting cell line with a suitable viability of over 90% was used. Combining the advantages of both enrichment systems using MACS to pre-enrich the stable cell line and FACS to isolate high-producing clones enabled to enrich 20.9-fold in this study, which comparable to the enrichment rate of two FACS subcloning steps. Analysis of MACS-enriched and FACS-sorted clones in a shake flask batch process led to titers of 130 mg/L. The stable starting cell line as well as the MACS-enriched stable cell line were also cultivated in this batch process both leading to comparable titers. As seen during the previous cultivation times of all MACS-enriched cell lines, the productivity dropped during the following cultivation period. Only using MACS does not lead to a stable high-producing cell line, but in combination with clone isolation it is a good tool to improve timelines as well as the amount of work of cell line development: A MACS enrichment step allows enrichment from $2x10^{10}$ cells in less than 2 hours with an additional week for expansion of cells. Using FACS it is only possible to screen about $1x10^{7}$ cells per hour and handling cell numbers of $2x10^{10}$ is not feasible (Philipps, 2004).

The batch results of up to 130 mg/L of the MACS-enriched and FACS-sorted clones are very promising. As seen before for clones isolated by limiting dilution, a further increase in maximal titer can to be expected for production in a fed-batch process. Several publications (Combs et al, 2011; Gorfien et al, 2003; Tabuchi et al, 2010, Strnad et al 2010) indicate that a further improvement in titer of about 2-fold are realistic for fed-batch production in bioreactors due to better control of temperature, culture pH, dissolved oxygen and continuous feed (Rodrigues et al, 2010).

A traditional cell line generation process using a DHFR amplification and clone isolation by limiting dilution takes at least 6 month (Butler, 2005; Wurm, 2004; Haines, 2009). Using the

non-amplifiable P5CS selection system in combination with a MACS-enrichment and clone isolation by FACS reduces the timeline to find a suitable clone to 3 month. In addition, the application of the tricistronic pMACS-CHO II vector for the expression of recombinant proteins coexpressing fluorescent reporter proteins is not limited to the use in combination with the P5CS selection system in CHO cells. The established system can be easily transferred to other selection markers and also other production cell lines.

5 Outlook

In the first part of the presented thesis the development of a novel selection system based on the enzyme pyrroline-5-carboxylate synthetase (P5CS) has been described. The P5CS selection system enables fast generation of stable cell lines expressing different recombinant proteins with high producer rates and can be combined with antibiotic selection systems for the use in a two-vector strategy. Neither during initial selection process nor during long-time cultivation of P5CS-selected cell lines any addition of cytotoxic drugs is necessary. Thereby, the P5CS selection system is optimal to modify parental CHO cell lines by overexpression of additional genes for modified glycosylation, ER secretion capacities or any other application. The currently reached titers of 140 mg/L in a fed-batch shake flask process are suitable for research applications, but not for high-level therapeutic protein production. To further improve the P5CS selection system, several approaches might be promising: The generation of a knock-down CHO cell line with deficiencies in both key enzymes for proline synthesis, OAT and P5CS, as well as a serum-free selection medium without traces of proline could greatly enhance the applied selection pressure and prevent recovery of non-producing cells. Furthermore, a combination of P5CS selection and a DHFR selection on one vector would allow an additional amplification step if required for further applications. A first test revealed an amplification of one vector and loss of the other. Therefore, the vector setting as well as the selection protocol would need to be improved. The amino acid proline has been reported to play an important role in protection of plant as well as mammalian cells against oxygen stress and overexpression of proline-forming enzymes has been proven to prevent reactive oxygen species (ROS)-induced apoptosis and to enhance cell survival. Apoptosis can account for a major part of cell death during the production process. Modified cell lines have shown improved performances in bioreactors. Analyzing the influence of the P5CS expression on the intracellular ROS levels and apoptosis might help to understand whether the endogenous proline synthesis increases apoptosis resistance.

In the second part of this thesis a tricistronic expression vector to enrich high-producers by the coexpression of fluorescent reporter proteins for both FACS as well as MACS was developed. The use of magnetic labeling allows a very fast and simple enrichment of high-producing cell lines. In addition, the clone selection by FACS has been proven to be superior compared to limiting dilution. The combination of MACS and FACS leads to clones with the same productivity as clones isolated by two FACS subcloning steps with an improved timeline and manual amount of work. One issue is the low correlation of secreted recombinant protein and cytosolic reporter protein. First experiments with membrane-bound fluorescent proteins processed in the secretory pathway as the secreted recombinant proteins already show an improved correlation. Therefore, switching both reporter proteins for recombinant antibody expression to membrane-bound reporter proteins seems to be very promising. For these two different surface markers, a MACS enrichment process with

releasable magnetic beads should be established to further improve the system by enabling enrichment of both antibody chains. The tricistronic expression vector is not limited for the use with the P5CS selection system in CHO cells. In general, an application in other expression hosts is possible and might be established.

6 Material and Methods

6.1 Materials

6.1.1 Technical devices

Analytical balances	CP224S	Satorius, Göttingen
	506-2	Kern, Balingen-Frommern
Autoclave	Varioklav® VI 500 EP	H+P Labortechnik, Oberschleißheim
	Systec V-95	Systec, Wettenberg
Blotting apparatus	Flastblot B34	Biometra, Göttingen
Cryo freezing container	Nalgene® Cryo 1°C Freezing Container Mr. Frosty®	Thermo Scientific, Bonn
Dispenser	Easypet®	Eppendorf, Hamburg
Electrophoresis apparatus	Horizon® 11.14	Biometra, Göttingen
	Horizon® 58	Biometra, Göttingen
	Xcell SureLock™	Invitrogen, Karlsruhe
	Consort E 865	Protrans, Hockenheim
	EPS 200	Amersham GE Healthcare, Freiburg
ELISA washer	HydroFlex™	Tecan, Männedorf
Flow cytometer	MACSQuant® Analyzer	Miltenyi Biotec, Bergisch Gladbach
	MACSQuant® VYB	Miltenyi Biotec, Bergisch Gladbach
	BD Influx™	Becton-Dickinson, Heidelberg
	FACSVantage™ SE DIVA	Becton-Dickinson, Heidelberg
Gele documentation	Gel Doc 1000	BioRad, München
Ice flaker	Scotsman AF 100	Hubbard Ice Systems, Ipswich
Incubator	Heracell® 240i	Heraeus, Hanau
	Heracell® BB6620	Heraeus, Hanau
	Heraeus® B6030	Heraeus, Hanau
	Binder C 150	Binder, Tuttlingen
Magnetic separator	OctoMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach
	thermoMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach
Microscope	Leica DM IL	Leica, Wetzlar
	Leica TCS SP2	Leica, Wetzlar
Mixer	Thermomixer compact	Eppendorf, Hamburg
	MACSmix™ Tube Rotator	Miltenyi Biotec, Bergisch Gladbach
	Vortex Genie	Scientific Industries, Portland
	C-MAG HS 4 IKAMAG®	IKA-Werke, Staufen
	GFL 3015	GFL, Burgwedel
PCR-cycler	Mastercycler® Gradient	Eppendorf, Hamburg
	AbiPrism® 7000	Applied Biosystems/Life Technologies, Darmstadt
pH meter	Labor-pH-Meter 765 Calimatic®	Knick, Berlin
Photometer	NanoDrop ND-1000	Thermo Scientific, Bonn
Pipet	Research® 10/100/200/1000 µL	Eppendorf, Hamburg
	Research® plus 8x 100/300 µL	Eppendorf, Hamburg
	Xplorer® 12x300 µL	Eppendorf, Hamburg
	HandyStep® Repeating Pipette	Brand, Wertheim
Plate reader	Emax	Molecular Devices, Biberach an der Riss
	Infinite® 200 PRO	Tecan, Männedorf

	VICTOR™ X4	PerkinElmer, Rodgau
Shake incubator	Certomat® CT plus	Satorius, Göttingen
	Forma Orbital Shaker	Thermo Scientific, Bonn
Sonificator	Sonoplus HD 2200	Brandelin electronic, Berlin
Sterile workbench	HERAsafe® HS	Thermo Scientific, Bonn
	HERAsafe® KSP	Thermo Scientific, Bonn
	Biowizard Golden Line	Kojar, Vilppula
Thermostat water bath	Type EC-5	Julabo, Seelbach
Water Purification	Milli-Q® Integral Water Purification System	Millipore/Merck, Darmstadt
Centrifuge	Heraeus® Multifuge 4Kr	Thermo Scientific, Bonn
	Heraeus® Biofuge Pico	Thermo Scientific, Bonn
	Centrifuge 5424	Eppendorf, Hamburg
	Centrifuge 5415 R	Eppendorf, Hamburg
	Centrifuge 5417 R	Eppendorf, Hamburg

6.1.2 Software & Databases

AbiPrism® 7000 SDS	PCR-cycler software	Applied Biosystems/Life Technologies, Darmstadt
ApE v2.0.15 to 45	Plasmid editor	Wayne Davis, University Utah, Salt Lake City
Clone Manager Professional version 9.x	Plasmid editor	Sci-Ed Software, Cary
Gel Doc 1000/PC	Gel Doc software	BioRad, München
i-control	Microplate reader software	Tecan, Männedorf
MACSQuantify Professional version 1.0	Analysis MACSQuant data	Miltenyi Biotec, Bergisch Gladbach
Microsoft Office version 12/14	Office suite	Microsoft, Redmont
NanoDrop 1000 V3.8.1	NanoDrop software	Thermo Scientific, Bonn
New WorkOut 2.5	Microplate reader software	PerkinElmer, Rodgau
SoftMax Pro	ELISA reader software	Molecular Devices, Biberach an der Riss
http://bioinfo.clontech.com/infusion/molarRatio.do	Cloning tool, insert: vector ratio	Clontech, Saint-Germain-en-Laye
http://blast.ncbi.nlm.nih.gov/Blast.cgi	Sequence blast	National Center for Biotechnology Information, Bethesda
http://www.ncbi.nlm.nih.gov/	Sequence and literature database	National Center for Biotechnology Information, Bethesda
http://www.promega.com/techserv/tools/biomath/calc11.htm#melt_results	Primer design	Promega, Mannheim
http://www.uniprot.org/	Sequence database	UniProt consortium

6.1.3 Consumables

4 - 20 % Tris-glycine-gel, 10 slots	Anamed Elektrophorese, Groß-Bieberau
ABsolute™ QPCR Seal	Thermo Scientific, Bonn
Amersham Hybond™ ECL™	GE Healthcare, Freiburg
Cell Strainer 40 μm	BD, Heidelberg
Conical centrifuge tube, 225 mL	BD, Heidelberg
Cryogenic vials 1.5/2.0 mL	Nalgene/Nunc, Langenselbold
Dispenser tips, Plastibrand PD-tips 1.25/2.5/5/12.5 mL	Brand, Wertheim
Erlenmeyer flask, vented cap, 125 mL	Corning, Hagen
Extra Thick Blot Paper	BioRad, München

Falcon tubes 15/50 mL	BD, Heidelberg
Filter pipet tip, ART, 1000 µL, sterile	Molecular Bio Products, San Diego
Filter pipet tip, Quali-tips 10/100/200 µL, sterile	Kisker Biotec, Steinfurt
Filter Unit Millex PVDF 0.22 µm	Millipore/Merck, Darmstadt
Immuno-plates MaxiSorp® 96-well	Nunc, Langenselbold
Latex glove Kimtech PFE	KIM Jarolim, Sulzdorf
Microcentrifuge tubes 1.5/2.0 mL	Starlab, Hamburg
Microtiter plate pureGrade™, 96-well, PS, V-bottom	Brand, Wertheim
Neubauer Improved	Brand, Wertheim
Nitrile gloves Supreno SE	Microflex, Reno
PCR tubes strip, 8x 0.2 mL, individual flat cap	Brand, Wertheim
Petri dish	Greiner Bio-One, Frickenhausen
Pipet tip, TipOne 10/200/1000 µL	Starlab, Hamburg
Pre-separation filters, 30 µm	Miltenyi Biotec, Bergisch Gladbach
Scalpel blades	Bayha, Tuttlingen
Separation columns MS	Miltenyi Biotec, Bergisch Gladbach
Serological pipettes 5/10/25 mL, unsterile	Sarstedt, Nümbrecht
Serological pipettes 5/10/25/50 mL, sterile	Sarstedt, Nümbrecht
Storage bottle 250/500/1000 mL	Corning, Hagen
Syringe, 1/5/10/50 mL, Plastipak	BD, Heidelberg
Thermo-Fast® 96 PCR detection plate	Thermo Scientific, Bonn
Tissue culture flask, 25 cm^2, screw cap with filter	TPP, Trasadingen
Tissue culture flask, 75/175 cm^2, vented cap	BD, Heidelberg
Tissue-culture plates 6-/12-/24-/96-well, flat bottom	BD, Heidelberg

6.1.4 Chemicals

2-propanol, molecular biology grade	AppliChem, Darmstadt
Acetic acid, 100%	AppliChem, Darmstadt
Agarosis, electrophoresis grade	Invitrogen, Karlsruhe
Ampicillin, sodium salt	AppliChem, Darmstadt
Bromphenol blue	Sigma-Aldrich, München
BSA fraction V	Serva Electrophoresis, Heidelberg
BSA Stock Solution, 10%	Miltenyi Biotec, Bergisch Gladbach
cOmplete Mini Protease Inhibitor Cocktail Tablets	Roche, Penzberg
Developer solution, Roentogen liquid	Tetenal, Norderstedt
EDTA, molecular biology grade	Sigma-Aldrich, München
Erythrosine B	Sigma-Aldrich, München
Ethanol, molecular biology grade	AppliChem, Darmstadt
Ethidium bromide solution, molecular biology grade	Sigma-Aldrich, München
Fixation solution, Roentogen Superfix	Tetenal, Norderstedt
Glycerin, molecular biology grade	Sigma-Aldrich, München
Glycine	Sigma-Aldrich, München
H_2SO_4, 95-97 % analysis grade	AppliChem, Darmstadt
HCl, 37%	Carl Roth, Karlsruhe
Immubilon™ Western Chemiluminescent HRP Substrate	Millipore, Schwalbach/Ts.
Kanamycinsulfat	AppliChem, Darmstadt
KCl, cell culture grade	AppliChem, Darmstadt
KH_2PO_4, cell culture grade	AppliChem, Darmstadt
MACSQuant Running Buffer	Miltenyi Biotec, Bergisch Gladbach
MACSQuant Storage Solution	Miltenyi Biotec, Bergisch Gladbach
MACSQuant Washing Solution	Miltenyi Biotec, Bergisch Gladbach
Methanol, analysis grade	AppliChem, Darmstadt
Milk powder	Sigma-Aldrich, München
Na_2HPO_4, cell culture grade	AppliChem, Darmstadt
NaCl, analysis grade	AppliChem, Darmstadt
NaOH, analysis grade	VWR, Darmstadt
Peptone, from soy bean, for microbiology	Merck, Darmstadt
Propidium iodide	Sigma-Aldrich, München
SDS running buffer	Anamed Elektrophorese, Groß-Bieberau
SDS, biochemistry grade	Carl Roth, Karlsruhe
Select agar	Sigma-Aldrich, München
SeramunBlau® fast TMB Substrate	Seramun, Heidesee

SOC	New England Biolabs, Frankfurt am Main
Sodium Deoxycholate	Sigma-Aldrich, München
Tris (Trizma base), molecular biology grade	Sigma-Aldrich, München
Triton X-100, laboratory grade	Sigma-Aldrich, München
Tween 20	Sigma-Aldrich, München
Water, molecular grade	Sigma-Aldrich, München
Yeast extract, for biotechnology	Merck, Darmstadt
β-mercaptoethanol, molecular biology grade	Sigma-Aldrich, München

6.1.5 Buffers & Solutions

Agarosis gel loading buffer (6x)	10% (v/v) 50x TAE buffer, 25% (v/v) glycerin, 0.2% (w/v) bromphenol blue
Ampicillin solution (1000x)	50 mg/mL ampicillin
ELISA Assay buffer	PBS, 1% (w/v) BSA
Erythrosine B solution (2x)	1 mg/mL in 1x PBS
Ethidium bromide solution (100x)	50 μg/mL ethidium bromide
Kanamycin solution (1000x)	50 mg/mL kanamycin
Laemmli buffer (5x)	250 mM Tris pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerin, 0.025% (w/v) bromphenol blue, 3.3% (v/v) β-mercaptoethanol
LB-agar	1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl
LB-agar plates	1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agarosis
MSX	1 mM, use aliquot immediately, do not refreeze
MTX	25 mM MTX
PBS	137 mM NaCl, 8.1 mM Na_2HPO_4, 2.6 mM KCl, 1.4 mM KH_2PO_4
PBST	PBS, 0.05 % (v/v) Tween 20
PE	PBS, 2 mM ETDA
PEB	PBS, 2 mM ETDA, 0.5 % (w/v) BSA
Propidium iodide solution (1000x)	1 mg/mL propidium iodide
RIPA	150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1%(w/v) SDS, 50 mM Tris pH 8.8, 1 % (v/v) Triton X-100, 1x protease inhibitors
TAE	40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.5
TBS	150 mM NaCl, 50 mM Tris, pH 7.0
TBST	TBS, 0.1% (v/v) Tween 20
Western Blot blocking buffer	TBS, 5% (w/v) milk powder
Western Blot transfer buffer	48 mM Tris, 39 mM glycine, 1.3 mM SDS, 10% (v/v) methanol

If not stated otherwise buffers were made with pure water.

6.1.6 Buffer, media and supplements for cell culture

6.1.6.1 Reagents

10x MEM (proline-free)	Sigma-Aldrich, München
BSA 30% solution	PAA, Cölbe
CD FortiCHO™	Invitrogen, Karlsruhe
Chemically defined lipid concentrate (1000x)	Invitrogen, Karlsruhe
CHO MACS CD	Miltenyi Biotec, Bergisch Gladbach
CHO MACS CD without proline	TeutoCell, Bielefeld
CHO MACS Feed Supplement	Miltenyi Biotec, Bergisch Gladbach
Dialyzed FBS	PAA, Cölbe
DL-methionine, insect cell culture grade	Sigma-Aldrich, München
DMSO, cell culture grade	Sigma-Aldrich, München
Erythrosine B	Sigma-Aldrich, München
ExCell® CD CHO	Sigma-Aldrich, München
FugeneHD®	Roche, Penzberg
G418 sulphate solution (50 mg/mL)	PAA, Cölbe
Genetix CloneMatrix	Molecular Devices, Biberach an der Riss
Gibco® FreeStyle™ CHO	Invitrogen, Karlsruhe
Glycine, cell culture grade	Sigma-Aldrich, München
HT supplement (100x)	Invitrogen, Karlsruhe
HyClone SFM4CHO (contains 4 mM glutamine)	Thermo Scientific, Bonn
Hygromycin B solution (50 mg/mL)	PAA, Cölbe
ITS	PAA, Cölbe
ITS+3	Sigma-Aldrich, München
L-alanine, cell culture grade	Sigma-Aldrich, München
L-arginine monohydrochloride, cell culture grade	Sigma-Aldrich, München
L-asparagine monohydrate, cell culture grade	Sigma-Aldrich, München
L-aspartic acid, cell culture grade	Sigma-Aldrich, München
L-cysteine hydrochloride, cell culture grade	Sigma-Aldrich, München
L-glutamic acid, cell culture grade	Sigma-Aldrich, München
L-glutamine (200 mM)	PAA, Cölbe
L-isoleucine, cell culture grade	Sigma-Aldrich, München
L-leucine, cell culture grade	Sigma-Aldrich, München
L-lysine monohydrochloride, cell culture grade	Sigma-Aldrich, München
L-methionine sulphoximine	Sigma-Aldrich, München
L-phenylalanine, cell culture grade	Sigma-Aldrich, München
L-proline, cell culture grade	Carl Roth, Karlsruhe
L-serine, cell culture grade	Sigma-Aldrich, München
L-threonine, cell culture grade	Sigma-Aldrich, München
L-tryptophan, cell culture grade	Sigma-Aldrich, München
L-tyrosine disodium salt hydrate, cell culture grade	Sigma-Aldrich, München
L-valine, cell culture grade	Sigma-Aldrich, München
MEM (proline-free)	Sigma-Aldrich, München
L-MSX	Sigma-Aldrich, München
MTX	Sigma-Aldrich, München
Penicillin/Streptomycin	PAA, Cölbe
Pluronic® F-68 solution, 10%	Sigma-Aldrich, München
RPMI Vitamins Solution (100x)	PAA, Cölbe
Sodium bicarbonate solution, 7.5 %	Sigma-Aldrich, München
Sodium pyruvate (100 mM)	PAA, Cölbe
Trace Elements A (1000x)	Cellgro, Manassas
Trace Elements B (1000x)	Cellgro, Manassas
TrypLE™ Select (1x)	Invitrogen, Karlsruhe
Trypsin	PAA, Cölbe
Zeocin™solution (100 mg/mL)	Invivogen, Toulouse

6.1.6.2 Media

MEM	MEM +/- 40 mg/L proline, 2 mM glutamine, 10% (v/v) dialyzed FBS
Serum-reduced MEM	MEM +/- 40 mg/L proline, 2 mM glutamine, 1:100 ITS+3, 5% (v/v) dialyzed FBS
CHO-MEM	MEM +/- 40 mg/L proline, 6 mM glutamine, 5% (v/v) dialyzed FBS, 1:100 ITS, 3 mM sodium pyruvate, 1x amino acid mixture, 1x RPMI vitamins solution, , 1x trace elements A and B, 1 % (v/v) Pluronic F-68, 2x chemically defined lipid concentrate, 0.05% (w/v) BSA
Amino acid mixture (50x)	0.85 g/L L-alanine, 4.25 g/L L-arginine hydrochloride, 5.5 g/L L-asparagine monohydrate, 1.75 g/L L-aspartic acid. 1.75 g/L L-cysteine hydrochloride, 4 g/L glutamine acid, 3 g/L glycine, 5.25 g/L L-isoleucine, 5.25 g/L L-leucine, 2.5 g/L L-lysine monohydrochloride, 1.05 g/L DL-methionine, 0.9 g/L L-phenylalanine, 2.6 g/L L-serine, 1.45 g/L L-threonine, 0.25 g/L L-tryptophan, 0.65 g/L L-tyrosine disodium salt hydrate, 1.2 g/L L-valine in water, sterile filtered
CHO MACS CD without proline	CHO MACS CD without proline, 6-8 mM glutamine, (8 mM batch, 6 mM fed-batch)
CHO MACS CD	CHO MACS CD, 8 mM glutamine
ExCell CD CHO	ExCell CD CHO, 8 mM glutamine
CD FortiCHO	CD FortiCHO, 8 mM glutamine
Gibco FreeStyle CHO	Gibco FreeStyle CHO, 8 mM glutamine
2x MEM	20% (v/v) 10x MEM, 0.45% (w/v) sodium bicarbonate, 20% (v/v) dialyzed FBS, sterile filtered 0.22 μm
ClonePix base	50 mL 2x MEM, 40 mL Genetix Clone Matrix
ClonePix medium	ClonePix base supplemented with 2 mM glutamine, 400 μg/mL G418 and 1:100 CloneDetect Agent

For the cultivation of CHO DG 44 cells, HT supplement (1x) was added to the media.

6.1.7 Cells

6.1.7.1 Bacterial cells

dam$^-$/dcm$^-$ Competent *E. coli*	New England Biolabs, Frankfurt am Main
NEB 5-alpha Competent *E. coli* (High Efficiency)	New England Biolabs, Frankfurt am Main

6.1.7.2 Mammalian cells

CHO-S	Invitrogen, Karlsruhe
CHO-K1 (ATCC-CCL-61)	American Type Culture Collection, Manassas
CHO DG44	Invitrogen, Karlsruhe
HEK EBNA (ATCC-CRL-10852)	American Type Culture Collection, Manassas

6.1.8 Enzymes

Antarctic phosphatase	New England Biolabs, Frankfurt am Main
Eurogenec qPCR Core kit for SYBR® Green I	Eurogentec, Liège
MACSscript	Miltenyi Biotec, Bergisch Gladbach
PicoMaxx High Fidelity PCR System	Agilent Technologies, Böblingen
Quick Blunting Kit™	New England Biolabs, Frankfurt am Main
Rapid DNA Ligation Kit	Roche, Penzberg
Restriction endonucleases	New England Biolabs, Frankfurt am Main

6.1.9 Others Kits and materials for molecular and biochemical applications

2-Log DNA Ladder (0.1–10.0 kb)	New England Biolabs, Frankfurt am Main
BCA™ Protein Assay Kit	Thermo Scientific, Bonn
BSA standard pre-diluted set	Thermo Scientific, Bonn
dNTPs (10 mM)	Fermentas, St. Leon-Rot
DTT (0.1 M)	Invitrogen, Darmstadt
FcR Blocking reagent	Miltenyi Biotec, Bergisch Gladbach
First strand buffer (5x)	Invitrogen, Darmstadt
High Pure PCR Product Purification Kit	Roche, Penzberg
Human/Mouse TGF beta 1 ELISA Ready-SET-Go!® (2nd Generation)	eBioscience, Frankfurt am Main
Inside Staining Kit	Miltenyi Biotec, Bergisch Gladbach
Invisorb® Spin Plasmid Mini Two	Stratec Molecular, Berlin
Low Molecular Weight DNA Ladder	New England Biolabs, Frankfurt am Main
NucleoBond® Xtra Maxi EF	Macherey-Nagel, Düren
PageRuler™ Prestained Protein Ladder	Fermentas, St. Leon-Rot
pGEM®-T Easy Vector System	Promega, Mannheim
Primer mix (as)	Miltenyi Biotec, Bergisch Gladbach
RNeasy® Mini Kit	Qiagen, Hilden
µMACS™ One-step cDNA Kit	Miltenyi Biotec, Bergisch Gladbach

6.1.10 Vectors

6.1.10.1 Commercial vectors & vectors from collaborations

pTagRFP-N	Source of RFP sequence, used as control vector	Evrogen, Moscow
pEGFP-C1	Source of GFP and neoR sequence, used as control vector	Clontech, Saint-Germain-en-Laye
pMACS 4-IRES.II	Backbone of pMACS-CHO	Miltenyi Biotec, Bergisch Gladbach
pGEM®-T Easy	Cloning vector for PCR products	Promega, Mannheim
pSV2neo-hCMV	Backbone of pSV2neo vector with hCMV promoter, backbone for pSV2neo-hCMV-GFP	Busch, 2005
pDisplay-GFP	Source of membrane-bound GFP sequence, used as control vector	Knöbel, 2006

6.1.10.2 Constructed vectors

6.1.10.2.1 Vectors with pSV2neo-hCMV backbone

Table 13: Vectors with pSV2neo-hCVM backbone

Vector	1. GOI hCMV promoter	2. GOI SV40 promoter
pSV2neo-hCMV-GFP	GFP	neoR

6.1.10.2.2 Vectors with pGEM-T Easy backbone

Table 14: Vectors with pGEM-T Easy backbone

Vector	Insert
pGEM-GAPDH	CHO GAPDH ORF
pGEM-OAT	CHO OAT ORF first 939 bp
pGEM-P5CS	CHO P5CS ORF
pGEM-GAPDH	CHO GAPDH ORF
pGEM-GS	CHO GS ORF
pGEM-mGFP F2A	mGFP for F2A vector (pMACS-CHO 2A)
pGEM-zeoR F2A	zeoR for F2A vector (pMACS-CHO 2A)
pGEM-GFP T2A	GFP for T2A vector (pMACS-CHO II)
pGEM-mGFP T2A	mGFP for T2A vector (pMACS-CHO II)
pGEM-RFP T2A	RFP for T2A vector (pMACS-CHO II)
pGEM- zeoR T2A	zeoR for T2A vector (pMACS-CHO II)
pGEM- P5CS T2A	P5CS for T2A vector (pMACS-CHO II)

6.1.10.2.3 Vectors with pMACS-backbone

Table 15: Vectors with pMACS-backbone

Vector	Backbone	1. GOI before IRES	2. GOI behind IRES	2A peptide	3. GOI behind 2A peptide	Additional elements
pAS-5	pMACS-CHO	GFP	-	-	-	
pAS-8	pMACS-CHO	GFP	neoR	-	-	
pAS-9	pMACS-CHO	-	GFP	-	-	
pAS-16	pMACS-CHO	GFP	P5CS	-	-	
pAS-17	pMACS-CHO	GFP	GS	-	-	
pAS-18	pMACS-CHO	GFP	hygroR	-	-	
pAS-19	pMACS-CHO	hum anti-CD303 HC	neoR	-	-	
pAS-20	pMACS-CHO	hum anti-CD303 HC	hygroR	-	-	
pAS-21	pMACS-CHO	hum anti-CD303 HC	P5CS	-	-	
pAS-23	pMACS-CHO	hum anti-CD303 LC	neoR	-	-	
pAS-24	pMACS-CHO	hum anti-CD303 LC	hygroR	-	-	
pAS-25	pMACS-CHO	hum anti-CD303 LC	P5CS	-	-	
pAS-31	pMACS-CHO	hum anti-CD303 HC	GFP	-	-	
pAS-32	pMACS-CHO	hum anti-CD303 LC	GFP	-	-	
pAS-46	pMACS-CHO	hum anti-CD303 HC	GS (CHO-K1)	-	-	
pAS-47	pMACS-CHO	hum anti-CD303 LC	GS (CHO-K1)	-	-	
pAS-54	pMACS-CHO	hum anti-CD303 HC	zeoR	-	-	
pAS-55	pMACS-CHO	hum anti-CD303 LC	zeoR	-	-	
pAS-83	pMACS-CHO-2A	hum anti-CD303 LC	neoR w/o Stop	F2A	mGFP	
pAS-84	pMACS-CHO-2A	hum anti-CD303 LC	neoR w/o Stop	F2A	RFP	
pAS-85	pMACS-CHO-2A	hum anti-CD303 HC	P5CS w/o Stop	F2A	mGFP	
pAS-86	pMACS-CHO-2A	hum anti-CD303 HC	P5CS w/o Stop	F2A	RFP	
pAS-97	pMACS-CHO	chim anti-Biotin HC	neoR	-	-	

pAS-98	pMACS-CHO	chim anti-Biotin HC	P5CS	-	-	
pAS-100	pMACS-CHO	chim anti-Biotin LC	zeoR	-	-	
pAS-102	pMACS-CHO	chim anti-CD14 HC	neoR	-	-	
pAS-103	pMACS-CHO	chim anti-CD14 HC	P5CS	-	-	
pAS-105	pMACS-CHO	chim anti-CD14 LC	zeoR	-	-	
pAS-107	pMACS-CHO-2A	hum anti-CD303 LC	zeoR w/o Stop	F2A	mGFP	
pAS-125	pMACS-CHO II	hum anti-CD303 LC	GFP w/o Stop	T2A	zeoR	
pAS-126	pMACS-CHO II	hum anti-CD303 LC	mGFP w/o Stop	T2A	zeoR	
pAS-130	pMACS-CHO II	hum anti-CD303 LC	RFP w/o Stop	T2A	zeoR	
pAS-131	pMACS-CHO II	hum anti-CD303 HC	GFP w/o Stop	T2A	P5CS	
pAS-132	pMACS-CHO II	hum anti-CD303 HC	mGFP w/o Stop	T2A	P5CS	
pAS-133	pMACS-CHO II	hum anti-CD303 HC	RFP w/o Stop	T2A	P5CS	
pAS-158	pMACS-CHO II	hum anti-CD303 LC	RFP w/o Stop	T2A	zeoR	DHFR with SV40 promoter
pJN-1	pMACS-CHO	hTGFβ1-FGA SP	P5CS	-		
pJN-2	pMACS-CHO	hTGFβ1-FGA SP	neoR	-		
pJN-3	pMACS-CHO	hTGFβ1-FGA SP	hygroR	-		
pJN-4	pMACS-CHO	hTGFβ1-FGA SP	zeoR	-		
pJN-5	pMACS-CHO II	hTGFβ1-FGA SP	GFP	T2A	P5CS	
pJN-6	pMACS-CHO II	hTGFβ1-FGA SP	mGFP	T2A	P5CS	

6.1.11 Oligonucleotides

Table 16: List of used oligonucleotides

Name	Sequence (5´→3´)	Annealing temperature	Application
OAT deg f	CRYVATGYTYTCYARRYTAGC	55°C	P
OAT deg r	CTCAGAARGACARGAYRGTC	55°C	P
OAT 3	ATGAATACAGGAGTGGAGGC	55°C	P, S
OAT 4 r	GGTCAGCATTATCTCATCATCAC	55°C	P, S
OAT 5 r	GAAGGACAAGATGGTCTTGTT	55°C	P
OAT 6 r	TGCAACAGATGCAGCAGAGG		S
OAT 7 r	GCCTTGGACTGTCTTCTTAG		S
P5CS deg f	AYATCDCAVSATGYTGAGWCA	55°C	P, S
P5CS deg r	TCARYTGRWRTKYCKCTGRGG	55°C	P, S
P5CS 2	GTACCCCTCAGTCGTGC	45°C	P, S
P5CS 4 r	CAGCCAGGCTATCATTATC	45°C	P, S
P5CS 7	GAACAAGTGACTGTCCCAAT	50°C	P, S
P5CS 8 r	GACGATGACATCTGTGTG	50°C	P, S
P5CS 11 r	AACGGCTCCGCTGGTGACC		S
P5CS 12 f	TCATCGATATGCTGCGAGTGG		S
OAT ORF f	ATGTTCTCCAGACTAGCAACTTTG	50°C	P
OAT ORF r	TCAGAAGGGGCAGATGGTC	50°C	P

P5CS ORF f	ATGTTGAGACACATGCATCG	55°C	P, S
P5CS ORF r	TCAGTTGGTGTTTCTCTGGG	55°C	P, S
GAPDH ORF f	ATGGTGAAGGTCGGCGTGAAC	50°C	P, S
GAPDH ORF r	TTACTCCTTGGAGGCCATGTAGG	50°C	P, S
GAPDH-84F	TGTTGCCATCAATGACCCCT	60°C	RT
GAPDH-184R	TCTCAGCCTTGACTGTGCCTT	60°C	RT
GAPDH-650F	AAGTCATCCCAGAGCTGAACG	60°C	RT
GAPDH-750R	AGGTTTCTCCAGGCGACATG	60°C	RT
OAT-825F	GCTGGCTGTGGATCATGAGA	60°C	RT
OAT-925R	CGTCATCACACAGCACTGCA	60°C	RT
OAT-670F	GTTGCTGCCTTCATGGTGG	60°C	RT
OAT-770R	TGGTGCCTGGTGCAGAGTT	60°C	RT
P5CS-519F	CGAGGCCATGTTTACGCAGT	60°C	RT
P5CS-619R	GTGTGCCGTTGAGATTCCG	60°C	RT
P5CS-920F	GCATGGAAGCCAAGGTGAA	60°C	RT
P5CS-1022R	GTGATGACGTGCCCAGACAC	60°C	RT
Seq IgG1	TTGGAGGGCTTATGGTTCAC		S
Seq kappa	CCTGGCTGTTTCCTGATTGC		S
GS ohne Tag f	GTGACT**CTCGAG**GCCGCCACCATGGCCA CCTCAGCAAGTTC	55°C	C
GS ohne Tag r	GTGACT**GGATCC**TTATCATTAGTTTTTGTA TTGAAGGGCTCG	55°C	C
Zeocin vor 2A f	GTGACT**CTCGAG**ATGGCGAAGCTTACTTC AGC	50°C	C
Zeocin vor 2 A r	GTGACT**TCTAGA**GTCCTGCTCCTCGGCCA CG	50°C	C
(m)GFP hinter F2A f	GTGACT**GCGATCGC**GAGACAGACACACT CCTGC	50°C	C
(m)GFP hinter F2A r	GTGACT**CTTAAG**ATGCTAACGTGGCTTCT TC	50°C	C
RFP hinter F2A f	GTGACT**GCGATCGC**ATGGTGTCTAAGGG CGAAGAG	50°C	C
RFP hinter F2A r	GTGACT**CTTAAG**TCAATTAAGTTTGTGCC CCAG	50°C	C
GFP vor T2A f	GTGACT**CTCGAG**GCCACCATGGTGAGCA AGGG	60°C	C
GFP vor T2A r	GTGACT**TCTAGA**CTTGTACAGCTCGTCCA TGCCG	60°C	C
mGFP vor T2A f	GTGACT**CTCGAG**GCCACCATGGAGACAG ACACAC	50°C	C
mGFP vor T2A r	GTGACT**TCTAGA**ACGTGGCTTCTTCTGCC AAAG	50°C	C
RFP vor T2A f	GTGACT**CTCGAG**GCCACCATGGTGTCTA AGGG	50°C	C
RFP vor T2A r	GTGACT**TCTAGA**ATTAAGTTTGTGCCCCA GTTTGC	50°C	C
T2A f	GTGACT**TCTAGA**TCTGGCTCCGGAGAGG GCC	60°C	C
T2A r	GTGACT**GCGATCGC**CGGGGCCGGGGTTC TCCTCC	60°	C
Zeocin hinter T2A f	GTGACT**GCGATCGC**GCGAAGCTTACTTC AGCGG	45°C	C
Zeocin hinter T2A r	GTGACT**CTTAAG**TCATCAGTCCTGCTCCT CGG	45°C	C
P5CS hinter T2A f	GTGACT**GCGATCGC**CTTAGGCACATGCA CAGATC	55°C	C
P5CS hinter T2A r	GTGACT**CTTAAG**TTATCAATGATGGTGGT GGTG	55°C	C
CMV-F	CGCAAATGGGCGGTAGGCGTG		S
pIRES-RP	TATAGACAAACGCACACCG		S
pcDNA1.1-RP	CTCTGTAGGTAGTTTGTCC		S
pcDNA-3.1-R	CAAACAACAGATGGCTGGC		S
M13 FP	TGTAAAACGACGGCCAGT		S
M13 RP	CAGGAAACAGCTATGACC		S

D = A or T or G, K = G or T, R = A or G, S = G or C, V = A or C or G, W = A or T, Y = C or T
S = sequencing, RT = real-time PCR, P = standard PCR (might be used for sequencing or cloning in pGEM®-T Easy vector), C = adding restriction enzyme sites for cloning in pGEM®-T Easy vector (addition of restriction enzymes sites in bold)

6.1.12 Antibodies

Table 17: List of used antibodies

Antibody	Distributor	Dilution	Application
Alexa Fluor® 647 Goat Anti-Rabbit IgG (H+L)	Invitrogen, Darmstadt	1:2000 in PEB	S
anti-human kappa HRP	In-house Miltenyi Biotec, Bergisch Gladbach	1:2000 – 1:8000	ELISA
Anti-Ig κ Light Chain-FITC, human	Miltenyi Biotec, Bergisch Gladbach	1:10 in PEB	ICS
Anti-IgG-APC, human	Miltenyi Biotec, Bergisch Gladbach	1:10 in PEB	ICS
Anti-LAP (TGF-β1)-APC, human	Miltenyi Biotec, Bergisch Gladbach	1:10 in PEB	ICS
Anti-Rabbit IgG Microbeads	Miltenyi Biotec, Bergisch Gladbach	1:10 in PEB	MACS
IgG (Fc) (anti- human, clone JDC-10)	Beckman Coulter, Krefeld	1:150 in PBS	ELISA
Labeling Check Reagent-APC	Miltenyi Biotec, Bergisch Gladbach	1:10 in PEB	S
Living Colors® Full-Length A.v. Polyclonal Antibody (polyclonal rabbit anti-GFP)	Clontech, Saint-Germain-en-Laye	1:1000 in PEB	MACS
Monoclonal anti-GAPDH HRP	Sigma-Aldrich, München	1:2500 in blocking buffer	WB
rabbit anti-ALDH18A1 (P5CS)	Sigma-Aldrich, München	1:1000 in TBST	WB
rabbit anti-OAT	Acris Antibodies, Herford	1:2500 in TBST	WB
Remicade®	MSD Sharp & Dohme, Haar	Standard, 0.976 to 250 ng/mL	ELISA
Stemgent® HRP mouse anti-rabbit	Miltenyi Biotec, Bergisch Gladbach	1:35000 in blocking buffer	WB
μMACS™ Anti-GFP MicroBeads (monoclonal)	Miltenyi Biotec, Bergisch Gladbach	1:10 in PEB	MACS
CloneDetect anti-human detection agent FITC-labeled	Molecular Devices, Biberach an der Riss	1:100	CP

S= surface cell staining, ICS = intracellular staining, ELISA = ELISA, WB= Western Blot, CP= ClonePix

6.2 Methods

6.2.1 Molecular biological methods

6.2.1.1 Isolation of plasmid DNA from *E.coli*

For small-scale isolation of plasmid DNA from *E.coli* overnight cultures, the Invisorb Spin Plasmid Mini Two kit and for large-scale isolation of plasmid DNA, the Macherey-Nagel

NucleoBond® Xtra Maxi EF kit was used according to manufacture´s instructions. Plasmid DNA concentrations were determined by absorption measurement.

6.2.1.2 Agarosis gel electrophoresis

Using an agarosis gel electrophoresis, linearized DNA can be separated according to their size. A 0.8-1.5% TAE agarosis gel containing 500 ng/mL ethidium bromide was loaded with the DNA mixed with 6x loading buffer. The sample DNA and the marker were separated at 10 V/cm^2 for 30 to 60 minutes depending on the DNA molecular weight and the length of the gel. The DNA bands separated in the gel were visualized by the intercalating dye ethidium bromide under UV light (λ= 312 nm). DNA bands were cut out and the DNA was purified with the Roche High Pure PCR Product Purification Kit.

6.2.1.3 Digestion of DNA by restriction endonucleases

Typ II restriction endonucleases enable the sequence specific cutting of DNA. DNA samples (500 ng - 10 μg) were digested with up to two NEB restriction endonucleases using corresponding reaction buffers at optimal temperatures (generally 37°C) with varying incubation times of one hour (h) to overnight depending on the specific requirements. For not compatible enzymes, the digest was purified (Roche High Pure PCR Product Purification Kit) and carried out in separate steps. After the incubation, restriction enzymes were heat-inactivated if possible.

6.2.1.4 Plasmid DNA purification

To purify PCR products, plasmid DNA from restriction enzyme digests or separated DNA from agarosis gels, the Roche High Pure PCR Product Purification Kit was used according to manufacturer´s instructions.

6.2.1.5 Blunting of digested DNA

For cloning of DNA fragments without compatible ends, DNA blunting was used to convert the incompatible 5´ or 3´overhangs to 5´ phosphorylated, blunt ends. The NEB Quick Blunting™ Kit was used according to manufacturer`s instruction following digestion. For restriction enzymes that could not be heat-inactivated, the digested DNA had to be purified in between.

6.2.1.6 Dephosphorylation of digested DNA

During DNA-dephosphorylation, the phosphate group at the 5´-end of the DNA is removed to avoid prevents religation of the vector. The dephosphorylation process carried out by the

NEB Antarctic Phosphatase according to manufacturer`s instruction was only performed, if the restriction enzymes produced compatible ends which would allow self-ligation. The used phosphatase can be completely inactivated by heat. Therefore, no purification step is necessary between dephosphorylation and ligation.

6.2.1.7 DNA ligation

DNA ligation was carried out with up to 200 ng DNA using the Roche Rapid DNA Ligation Kit at room temperature (RT) for 5 to 90 minutes (min). For ligation of ends with compatible overhangs ("sticky"), a molar ratio of vector to insert of 1:3 was chosen. For ligation of ends without overhangs ("blunt"), a molar ratio of vector to insert of 1:10 was used to increase probability of insert integration.

$$amount\ insert\ (ng) = \frac{amount\ vector\ (ng) * size\ insert\ (bp)}{size\ vector\ (bp)} * ratio\ (insert/vector)$$

For cloning of PCR products, the PCR product was isolated and purified by agarosis gel electrophoresis and gel extraction followed by a cloning step into the pGEM®-T Easy Vector System according to manufacturer's instructions.

6.2.1.8 Photometric determination of DNA concentrations

DNA has the nature to absorb UV light at 260 nm. Measuring the absorbtion at 260 nm enables to calculate DNA concentration according to the Beer Lambert Law:

Oligonucleotides:	$OD_{260\ nm} = 1$	$\approx 20\ \mu g$
Single-strand DNA:	$OD_{260\ nm} = 1$	$\approx 40\ \mu g/mL$
Double-strand DNA:	$OD_{260\ nm} = 1$	$\approx 50\ \mu g/mL$

In addition, the absobtion at 280 nm, which is the absorbtion maximum of proteins, can be determined to calculate the 260:280 ratio, which is commonly used to analyze protein contaminations. A ratio of 1.8 indicates a DNA preparation relatively free of protein contaminations.

6.2.1.9 PCR

DNA-fragments were amplified using Polymerase chain reaction (PCR) (Mullis et al, 1986). The used PicoMaxx High Fidelity PCR System (Agilent Technologies) makes use of two different thermostable polymerases, *Taq* and *Pfu*. Due to the ability of proofreading activity the *Pfu* polymerase ensures higher fidelity. The mixture with *Taq* polymerase is commonly used to compensate for the reduced speed of *Pfu* polymerase. Complementary oligonucleotides (primers) flanking the amplified region were synthesized by Metabion. If degenerated primers were used, the primer concentration was increased depending on the number of degenerated bases.

Standard reaction mixture:		
Template DNA	1 µL	5-50 ng
10x PCR buffer	10 µL	1x
Sense primer	1 µL	200 pmol/µL
Antisense primer	1 µL	200 pmol/µL
dNTPs	1 µL	200 µM per nucleotide
Polymerase	1 µL	0.05 U/µL
Water, molecular grade	ad 50 µL	

Standard PCR cycle:			
Initial denaturation	95 °C	1 min	
Denaturation	95 °C	1 min	
Annealing	as described for oligonucleotide	1 min	34x
Elongation	72 °C	1 min per 1 kb	
Final elongation	72 °C	10 min	
Hold	10 °C	∞	

6.2.1.10 Semi-quantitative real-time PCR

To compare cDNA expression levels, a semi-quantitative real-time PCR approach was chosen using the Eurogenec qPCR Core kit for SYBR® Green I. All samples were measured as triplicates:

Standard reaction mixture:		
Template DNA	3 µL	15 ng
10x PCR buffer	2 µL	1x
Sense primer	0.4 µL	200 nM
Antisense primer	0.4 µL	200 nM
dNTPs	0.8 µL	200 µM per nucleotide
Polymerase	0.1 µL	0.025 U/µL
$MgCl_2$	1.4 µL	3.5 mM
SYBRGreen (1:2000)	0.6 µL	
Water, molecular grade	ad 50 µL	

Standard PCR cycle:			
Pre-heating	50°C	2 min	
Initial denaturation	95 °C	10 min	
Denaturation	95 °C	15 sec	
Annealing and Elongation	60°C	1 min	39x
Mmelt curve	95°C → 60°C		

Target genes´ cDNA (OAT, P5CS) and the reference gene´s cDNA (GAPDH) were PCR amplified and cloned into the pGEM®-T Easy Vector System. Real-time PCR using dilutions of these control vectors indicate that all three genes have similar efficiencies allowing a ΔΔ-CT analysis.

6.2.1.11 DNA sequencing

For sequence verification, samples (plasmid DNA or PCR products) were sequenced at a commercial provider (GATC). DNA samples were dissolved in molecular grade water to a concentration of 50 ng/µL. Custom-made primers were diluted to a concentration of 10 pmol/µL.

6.2.1.12 Isolation of RNA from mammalian cells and cDNA synthesis

For real-time PCR applications RNA was isolated using the Qiagen RNeasy® Mini Kit followed by a cDNA synthesis protocol:

Standard reaction mixture:

RNA	1 µL	1 µg
Water, molecular grade	10 µL	
Primer mix (as)	1 µL	

1. Mix and incubate for 10 minutes at 70°C
2. Immediately cool down on ice
3. Add the following components:

First strand buffer (5x)	4 µL	1x
DTT (0.1 M)	2 µL	10 mM
dNTPs (10 mM)	1 µL	0.5 mM

4. Mix and incubate for 10 minutes at room temperature
5. Incubate 2 minutes at 40°C
6. Add the following components:

MACSscript	1 µL

7. Mix and incubate for 50 minutes at 42°C
8. Heat-inactivate 15 minutes at 70°C

For all other applications, mRNA isolation and subsequent cDNA synthesis was performed with the Miltenyi Biotec µMACS™ One-step cDNA Starting Kit according to manufacturer`s instruction.

6.2.1.13 Cultivation of *E.coli* for plasmid preparation

6.2.1.13.1 Transformation of *E.coli*

E.coli were transformed by heat-shock transformation. In general NEB 5-alpha competent *E.coli* (high efficiency) were used. If subsequently the plasmid was going to be digested with methylation-sensitive restriction enzymes, the NEB dam-/dcm- competent *E.coli* were used. Half of a ligation reaction or 1-5 ng of a plasmid solution were mixed with the 50 µL competent E.coli and incubated on ice for 5 to 30 minutes. The heat-shock of 30 seconds (sec) at 42°C in a water bath was followed by cooling down on ice and addition of SOC medium. For re-transfections or sticky ligation with ampicillin selection, bacteria were directly spread on LB-agar plates containing appropriate selection antibiotics (50 µg/mL amplcillin or 50 µg/mL kanamycin). For all other application, bacteria were first incubated for up to 1 hours shaken at 37°C. LB-plates were incubated overnight at 37°C.

6.2.1.13.2 Overnight cultures for plasmid preparation

A single colony from a LB-agar plate was transferred into LB-Medium with selection antibiotics and incubated overnight at 250 rpm (25 mm orbit) at 37°C in an Erlenmeyer flask with a volume five times larger than the culture volume.

6.2.2 Biochemical methods

6.2.2.1 Cell lysis

Harvested cells were lysed in RIPA (Radioimmunoprecipitation assay) buffer to enable the extraction of membrane, cytoplasmic and nuclear proteins. Up to $1x10^7$ cells harvested on ice were suspended in 1 mL ice-cold RIPA buffer containing protease inhibitors (Roche, Complete Protease Inhibitor Cocktail Tablets Mini) and were sonificated (3 times for 10 sec, 30% pulses) on ice avoiding foaming. The lysate was incubated 10 minutes on ice on a shaker followed by a centrifugation step (10 min, 14,000 xg, 4°C) to remove cell debris. The protein lysate was split into several aliquots to avoid freezing-thawing cycles and stored at -20°C.

6.2.2.2 Quantification of proteins

Protein concentrations were determined by BCA assay using the Pierce BCA™ Protein Assay Reagent and Bovine Serum Albumin (BSA) Standard Pre-Diluted Set in microtiter plates according to manufacturer´s instructions.

6.2.2.3 SDS-PAGE

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is used to separate proteins according to their size. Protein samples were mixed with 5x Laemmli sample buffer, heated to 95°C for 5 minutes and loaded on 4 – 20 % Tris-Glycine gels (Anamed). As a marker the PAGERuler™ Prestained Protein Ladder (Fermentas) and as running buffer a Tris-Glycine buffer (Anamed) was used. Gels were run at 200 V, 30 mA and 5 W per gel.

6.2.2.4 Western Blot

Using Western Blot, proteins separated by SDS-PAGE can be detected and identified by specific antibodies. The proteins separated by SDS-PAGE were transferred from the gel onto a nitrocellulose membrane (HyBond ECL, Amersham), which was pre-incubated in transfer buffer for 15 minutes. The nitrocellulose membrane was bedded on a blot paper soaked in transfer buffer in a semi-dry blotter. Avoiding air bubbles, the gel was put on top of the nitrocellulose membrane followed by another layer of soaked blot paper. By applying an electrical field (per gel 15 V, 100 mA, 2 W) for 30 minutes, proteins were transferred. After the transfer, membranes were washed to remove transfer buffer and, if blocking was

necessary for the used antibody, membranes were incubated for 1 hour at room temperature in blocking buffer. Incubations of membranes in antibody solutions were performed in the concentrations and buffers listed in Table 18 for 1 hour at room temperature. After each step, membranes were washed 3 times for 10 seconds and subsequently 3 times for 1 minute in TBST. Incubation and washing steps were performed using the MACSmix™ Tube Rotator.

Table 18: Antibodies used for Western Blots

Detected protein	Blocking step	Primary antibody		Secondary antibody	
P5CS	-	rabbit anti-ALDH18A1	in TBST	Stemgent® HRP mouse anti-rabbit	in blocking buffer
OAT	-	rabbit anti-OAT	in TBST	Stemgent® HRP mouse anti-rabbit	in blocking buffer
GAPDH	1h blocking buffer	-	-	monoclonal anti-GAPDH HRP	in blocking buffer

After immunolabeling, the washed membranes were incubated for 5 minutes with a HRP substrate (Immobilon™ Western Chemiluminescent HRP Substrate, Millipore). A photographic film was exposed for 10 seconds to several minutes to the membrane, followed by an incubation in developer solution (Roentogen, Tetenal), washing in water, incubation in fixation solution (Roentogen, Tetenal) and another washing step in water. Each incubation step was performed for 1 minute.

6.2.2.5 ELISA

Concentration of secreted recombinant proteins were measured by ELISA (enzyme linked immunosorbent assay). Maximal titers were determined by seeding cells into an additional cell culture flask or plate and letting these cells overgrow. Supernatants were collected once cells had reached 100% confluence and the majority of cells already showed signs of dying, commonly after 5 to 7 day. Supenatants were centrifuged (5 min, 3,000 xg, RT) and the titer was determined directly or supernatants were stored at -80°C.

6.2.2.5.1 Human IgG and kappa specific ELISA

Nunc MaxiSorb® 96-well microtiter plates were coated with 100 µL mouse anti-human IgG (FC) antibody (Beckman Coulter) (3.33 µg/mL) in PBS and incubated at 4°C overnight. The plate was washed with 300 µL PBST for 3 times and to block free binding sites 100 µL ELISA Assay buffer were added for 1 hour at 37°C. Again the plate was washed. A standard antibody dilution series (Remicade®, 0.976 to 250 ng/mL) was prepared in Assay Buffer. 100 µL per well of samples, supernatants of transfected cells, at different dilutions, blanks, recovery samples and the standard series were loaded as duplicates into the microtiter and incubated at 37°C for 1 hour. After washing, 100 µL horseradish peroxidase conjugated anti-

human kappa specific antibody (Miltenyi Biotec, in-house, 1:2.000 – 1:8.000 in Assay Buffer) was added and incubated at 37°C for 1 hour. After washing, 100 µL TMB substrate (SeramunBlau® fast) was added and developed at room temperature for about 2 minutes (time course depends on the color development). The reaction was stopped by adding 100 µL 1 N sulphuric acid and the absorbance at 450 nm was read with an ELISA reader.

6.2.2.5.2 TGF-β specific ELISA

For detection of human TGF-β1 the human/mouse TGF beta 1 ELISA Ready-SET-Go!® (eBioscience) was used according to the manufacturer´s protocol. In short Nunc MaxiSorb® 96-well microtiter plates were coated with an anti-TGF-β1 antibody, blocked and loaded with acid-activated samples, a standard series (0.62-16 ng/mL TGF-β1) and recovery samples. TGF-β1 was detected by a biotin-conjugated anti-TGF-β1 antibody and avidin-HRP followed by a 10 minute incubation at room temperature with TMB and stopping of the reaction with 1 N sulphuric acid. The absorbance at 450 nm was read with an ELISA reader.

6.2.2.6 Intracellular staining

$1x10^6$ cells were harvested as a single cell suspension, centrifuged (5 min, 300 xg, RT) and resuspended in 100 µL PEB. For fixation 400 µL Inside Fix (Miltenyi Biotec, Inside Stain Kit) was added and incubated for 30 minutes at room temperature. For washing, 3 mL Inside Perm (Miltenyi Biotec, Inside Stain Kit) were added and the cells were centrifuged (5 min, 300 xg, RT). The supernatant was discarded. Cells were resuspended in appropriate amounts of Inside Perm (90 µL for staining with one antibody, 80 µL for staining with two antibodies) and incubated for 20 minutes at room temperature to make the cells permeable. After 20 minutes, fluorescent dye-conjugated antibodies (Table 19) were added and incubated for another 20 minutes at room temperature in the dark. For washing, 1 mL Inside Perm was added, cells were centrifuged (5 min, 300 xg, RT) and the supernatant was discarded. Cells were resuspended in 1 mL PE and analyzed by flow cytometry using appropriate unstained and single-stained samples for compensation and gating.

Table 19: Antibodies used for intracellular stainings

Staining	Antibody
Human IgG/kappa antibody - staining	Anti-Ig κ Light Chain-FITC and human and Anti-IgG-APC, human
LAP-staining	Anti-LAP (TGF-β1)-APC, human

6.2.3 Cell culture methods

6.2.3.1 Cultivation of cells

6.2.3.1.1 Passaging of adherent cells

Adherent CHO cells were routinely cultured in appropriate media such as MEM or the modified CHO-MEM. The cells were incubated at 37°C in a humid CO_2 incubator in an atmosphere containing 5 % CO_2. CHO cells were passaged every 3 to 4 days before reaching confluency by washing with PBS and incubating with trypsin-EDTA until the cells detached from the bottom of the cell culture flask. The cells were then diluted in fresh serum containing culture medium. If the trypsin-EDTA concentration in the fresh medium was lower than 5%, no centrifugation step was necessary. Otherwise cells were centrifuged (10 min, 100 xg, RT), the supernatant was discarded and cells were resuspended in fresh medium to remove the trypsin-EDTA.

6.2.3.1.2 Cultivation of suspension cells

Suspension CHO cells were routinely cultured in appropriate serum-free suspension media at 37°C in a humid CO_2 incubator in an atmosphere containing 5 to 9 % CO_2 as static cultures or in an orbital shake incubator at 125 rpm (50 mm orbit). Every 2 to 3 days, cell number and viability were determined. To keep the cells in the logarithmic growth phase, cells were not allowed to grow to densities over $1x10^6$ cells/mL. Routinely, cells were diluted to $2x10^5$ cells/mL. During adaptation to serum-free suspension media or adaptation to the orbital shake incubator, cells were seeded at higher cell densities of 3 to $5x10^5$ cells/mL.

6.2.3.2 Cryo-conservation

For long-term storage, the cell lines were harvested as described above. $1x10^6$ to $1x10^7$ cells per cryo-vial were centrifuged (10 min, 100 xg, RT) and the supernatant was discarded. Cells were then resuspended in a cryo-medium consisting of 20 % FBS, 10% DMSO and 70% culture medium without antibiotics and transferred into 1.5 mL cryo-vial. Cryo-vials were immediately transferred into Cryo 1°C Freezing Containers (Nalgene®) to allow a slow and gradual freezing down to -80°C for 1-2 days. Then, the tubes were transferred to liquid nitrogen. For recultivation, cells were thawn in a 37°C water bath, transferred into culture medium and centrifuged (10 min, 100 xg, RT) to remove the DMSO. After resuspension in appropriate culture media, cells were cultivated as described.

6.2.3.3 Determination of cell number and viability

Cell numbers and viability were determined by manual counting in a Neubauer hemacytometer or automated counting using the MACSQuant® Analyzer. For counting in a

Neubauer hemacytometer cells were stained with Erythrosine B solution to differentiate between dead and living cells. Erythrosine B can only penetrate cells with damaged cytoplasm membranes and therefore, only dead cells are stained. Due to the defined volume in each square of the Neubauer hemacytometer, the cell concentration can be calculated as following:

$$concentration\ (mL^{-1}) = \frac{number\ of\ viable\ cells * dilution\ factor}{number\ of\ squares} * 10^4$$

The vitality can be calculated by the numbers of viable and total cells:

$$viability\ (\%) = \frac{number\ of\ viable\ cells}{number\ of\ all\ cells} * 100$$

Counting cells using the MACSQuant® analysis is only suitable for single cell suspension. To stain dead cells, 0.5 μL propidium iodide solution was added directly before measurement. Similar to Erythrosine B, propidium iodide can only penetrate cells with damaged cell membranes and the fluorescent dye then intercalates into the DNA. For cell counting, no forward/sideward scatter gate was set to include doublets into the counting and at least $2x10^4$ cells were counted at a flow rate lower than 1000 cells per second to increase accuracy.

6.2.3.4 Transfection

Several different chemical transfection reagents were tested and optimized for the used cell lines. Due to the best efficiencies and viabilities, adherent CHO cells were routinely transfected using the FugeneHD® transfection reagent. Optimal transfection efficiencies were reached by seeding the adherent CHO one day before transfection at cell densities of $1x10^5$ per 12-well plate (3.8 cm^2) in 1 mL culture medium. For transfection, 1 μg DNA in 100 μL culture medium without serum was mixed with 4 μL FugeneHD® and incubated for 15 minutes at room temperature. Per 12-well, 50 μL of this transfection mixture was added drop-wise onto the cells. For larger cell culture dishes and higher cell numbers the transfection mixture was upscaled. For transient transfections, cells were harvested 24 to 48 hours after transfection for analysis of expressed fluorescent protein or the supernatant was harvested 3 to 5 days after transfection for analysis of secreted recombinant proteins.

6.2.3.5 Selection of stable cell lines

For the generation of stable cell lines, CHO cells were transfected as described above. In first transfections, stable cell lines were generated with a mild split rates of 1:3 to 1:6 24 hours after transfection and addition of selection pressure 48 hours after transfection. A harsher protocol with split rates of 1:8 to 1:20 into a larger culture flask directly into selection medium 24 hours after transfection was used as a standard protocol. These thin cell densities

ensure most stringent selection conditions. For P5CS selection, the used selection medium did not contain proline. Appropriate concentrations of antibiotics for antibiotic selections, MSX for GS selections and MTX for DHFR mediated MTX amplification were added (Table 20).

Table 20: Used selection reagents

Selection reagent	Concentration in selection medium	
	CHO-S	CHO-K1
Neomycin (G418)	800 µg/mL	400 µg/mL
Zeocin[1M]	100 µg/mL	50 µg/mL
Hygromycin B	800 µg/mL	600 µg/mL
MTX	50/100/200 nM	-
MSX	25 µM	

To find appropriate concentrations, selection antibiotics and reagents were tested on untransfected cells. A suitable concentration leads to killing of cells in 7 to 10 days. Additionally, a determined range of concentration was tested during the stable cell line generation to optimize the concentrations. During selection, medium was changed once per week. Once cell started to grow up after selection, they were passaged and maintained as described in the appropriate selection medium.

6.2.3.6 Clone isolation using limiting dilution

To isolate clones, which are originated from one single cell to obtain a more homologous cell population, a single cell suspension of adherent CHO cells was filtered through a 40 nm cell strainer to remove any clumping cells. The cell suspension was counted in a Neubauer hemacytometer and diluted down to a concentration of 3 to 5 cells/mL. Dispensing 100 µL of this suspension per 96-well resulted in 0.3 to 0.5 cell/well. After about 10 days, 96-well plates were screened for formation of visible colonies by microscope. The supernatant of wells containing a colony was screended by ELISA for recombinant protein expression.

6.2.3.6.1 Clone isolation by clone picking from semi-solid media

To visualize secreted antibody the Genetix CloneDetect anti-human detection agent FITC-labeled was used. Filtered single cells were seeded at 0.3 to 0.5 cell/well in a 96-well plate in the usual selection medium. After 24 hours, the medium was exchanged to semi-solid ClonePix medium. Formed colonies were screened for fluorescence by microscope 19 days after seeding. High-fluorescent cells were expanded.

6.2.3.7 Flow cytometry analysis

Flow cytometry is used to identify, characterize and separate different types of cells based on detecting and measuring the fluorescence emitted after excitation with a laser. Cells were

analyzed in PEB or CliniMACS® buffer (Miltenyi Biotec) using the MACSQuant® Analyzer and Software. Cell populations were identified by their physical properties in the forward scatter (FSC) and sideward scatter (SSC). Gating on the analyzed population in the FCS and SSC blot together with and propidium iodide staining ensured fluorescence analysis of single, living cells. Stained or fluorescent cells were always compared to non-fluorescing negative sample. Single fluorescing samples were used to compensate overlapping fluorescence emission spectra.

6.2.3.8 Clone isolation by FACS sorting

Fluorescence activated cell sorting (FACS®) allows to isolate defined cell poplations identified by flow cytometry. To isolate high-producing clones, stably expressing cell lines were FACS sorted (BD Bioscience Influx™ Cell Sorter or BD Bioscience FACSVantage™ SE DIVA, at Fraunhofer IME, Aachen) according to the intensity of the coexpressed fluorescent protein. The 0.1 to 1 % brightest single or double positive cells were sorted as single cells into 96-well plates containing selection media. To improve cloning recovery, antibiotic concentration were reduced factor 2 and serum concentrations were increased from 5% to 10% during the first 5 days. After about 10 days, 96-well plates were screened for formation of visible colonies by microscope and recombinant protein expression by ELISA.

6.2.3.9 Cell enrichment by MACS

Magnetic-activated cell sorting (MACS®) enables the enrichment of specific cells using surface markers. First, the surface marker is directly or indirectly labeled with a conjugate of antibody and supermagnetic particle (microbead). The cell mixture is then separated on a column in a magnetic field. Labeled cells are retained and can be eluted from the column once the magnetic field is removed.

6.2.3.9.1 Immunolabeling for MACS separation

The surface marker used as target for magnetic enrichment was the membrane-bound GFP. Up to $1x10^7$ cells in a single cell suspension, detached by TrypLE to reduce trypsin dependent degradation of the surface marker, were labeled indirect with a polyclonal rabbit-anti-GFP antibody and anti-rabbit IgG MicroBeads or labeled directly with a monoclonal anti-GFP coupled to microbeads (Table 21) according to Miltenyi Biotec´s staining protocol.

Table 21: Antibodies used for MACS enrichment

	Direct staining	**Indirect staining**
First antibody	µMACS™ Anti-GFP Microbeads (monoclonal)	Living Colors® Full-Length A.v. Polyclonal Antibody (polyclonal rabbit anti-GFP)
Second antibody	-	Anti-Rabbit IgG MicroBeads
Labeling check	Labeling Check Reagent-APC	Labeling Check Reagent-APC

To avoid false-positive staining due to Fc receptor interactions, the mouse FcR Blocking Reagent was applied prior to antibody labeling. To reduce back round staining all steps were performed on ice and cells were washed twice after each labeling step. To control the specificity of the antibody staining, microbeads can be stained with the Labeling Check Reagent-APC according to the manufacturer's instructions.

6.2.3.9.2 MACS enrichment

Magnetic-labeled cells were loaded on a 4°C pre-cooled MS column placed in the magnetic field of an OctoMACS™ Separator (Miltenyi Biotec). The column was washed to remove GFP-negative and low expressing cells. Magnetically labeled high-positive GFP cells were retained within the column and eluted as the positively selected cell fraction after removing the column from the magnet. To increase stringency, the eluted cells can be loaded on a second column. There, washing and elution is identical to the first column. Separation efficiency is monitored by GFP expression in the original, wash and elution fraction using MACSQuant® Analyzer (Miltenyi Biotec). Eluted cells are cultured in the described selection medium.

6.2.3.10 Test productions in orbital shake incubator

6.2.3.10.1 Batch process

Clones producing recombinant proteins adapted to growth in serum-free medium and the orbital shake incubator from the logarithmic growth phase were seeded at cell densities of $2x10^5$ cells/mL in 30 mL medium and incubated at 37°C in a humid CO_2 shake incubator in an atmosphere containing 5 to 9 % CO_2 at 125 rpm (50 mm orbital). Each day a 0.5 mL aliquot was taken to determine the cell number using the MACSQuant® Analyzer and the recombinant protein concentration in the supernatant by ELISA.

6.2.3.10.2 Fed-batch process

The fed-batch process is identical to the described batch process with the difference of a daily feed with CHO MACS Feed Supplement. Depending on the counted cell numbers, the feeding amount was increased: The feed was started with 3.1 % of culture medium once cell numbers of over $1x10^6$ cells/mL were reached. At over $5x10^6$ cells/mL, it was increased to 7.5% for two days and to 8% for the remaining culture time (Table 22).

Table 22: Feed during fed-batch culture

	Feed % of culture volume	
Day	Slower growing clones	Faster growing clones
0	0	0
1	0	0
2	0	0
3	3,1	3,1
4	3,1	3,1
5	3,1	7,5
6	7,5	7,5
7	7,5	7,5
8	7,5	8
> 9	8	8

6.2.3.10.3 Specific productivity

To quantitatively compare different clones, the cell specific productivity is calculated by the ration of integral viable cell density (IVCD) and produced recombinant protein concentration. The integral of viable cell density (IVCD) is the most frequently used method to compare the average viable cell density and is represented by the area under the curve when the concentration of viable cells is plotted against the culture time. To calculate the IVCD, the trapezoidal rule is used as an approximation technique (Adams et al, 2007).

The specific productivity can be determined by graphically plotting the recombinant protein expression against the IVCD of the logarithmic growth phase (Figure 56). The resulting slop equates to the specific productivity.

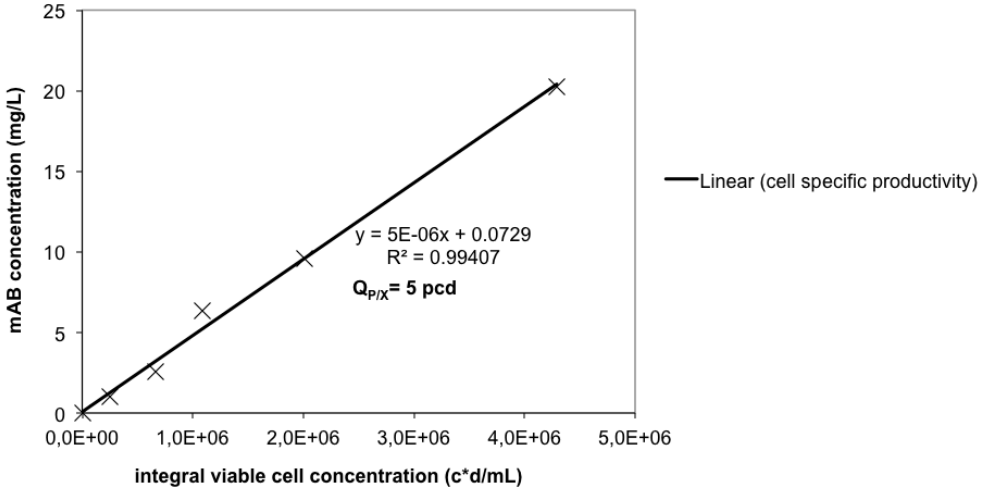

Figure 56: Determination of specific productivity

The specific productivity can be determined by graphically plotting the recombinant protein expression against the IVCD of the logarithmic growth phase. The resulting slop equates to the specific productivity.

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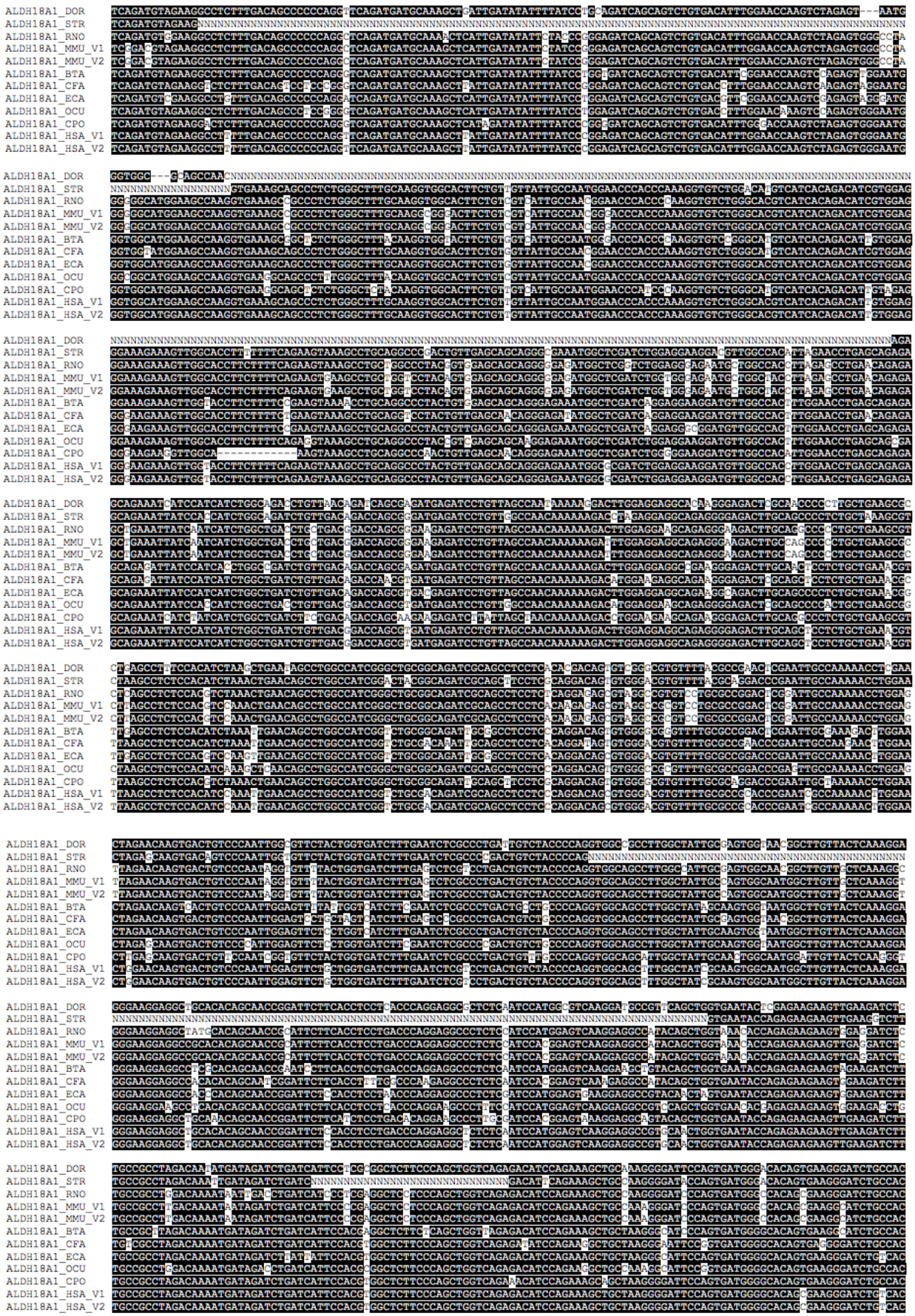
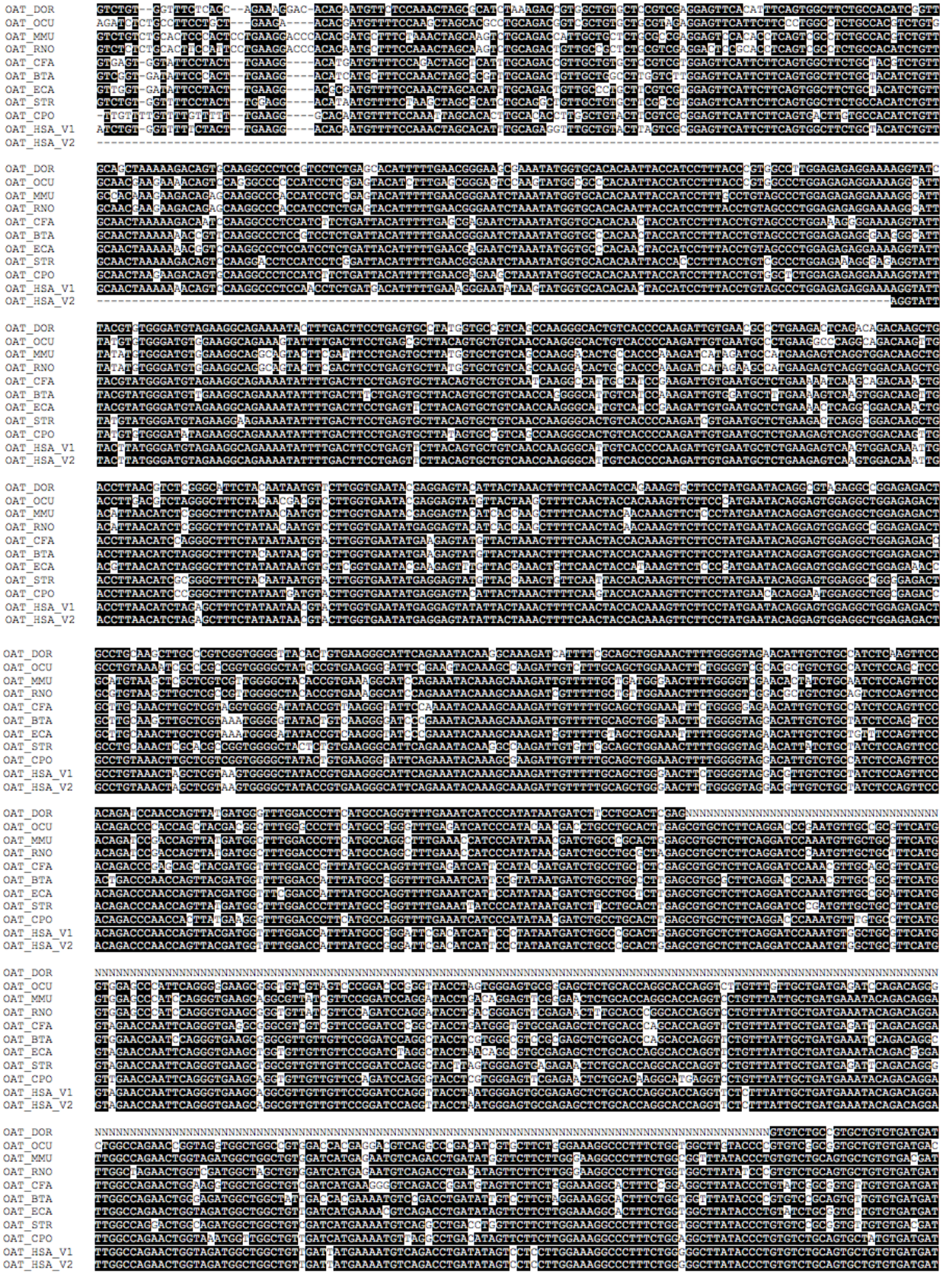
8 Appendix

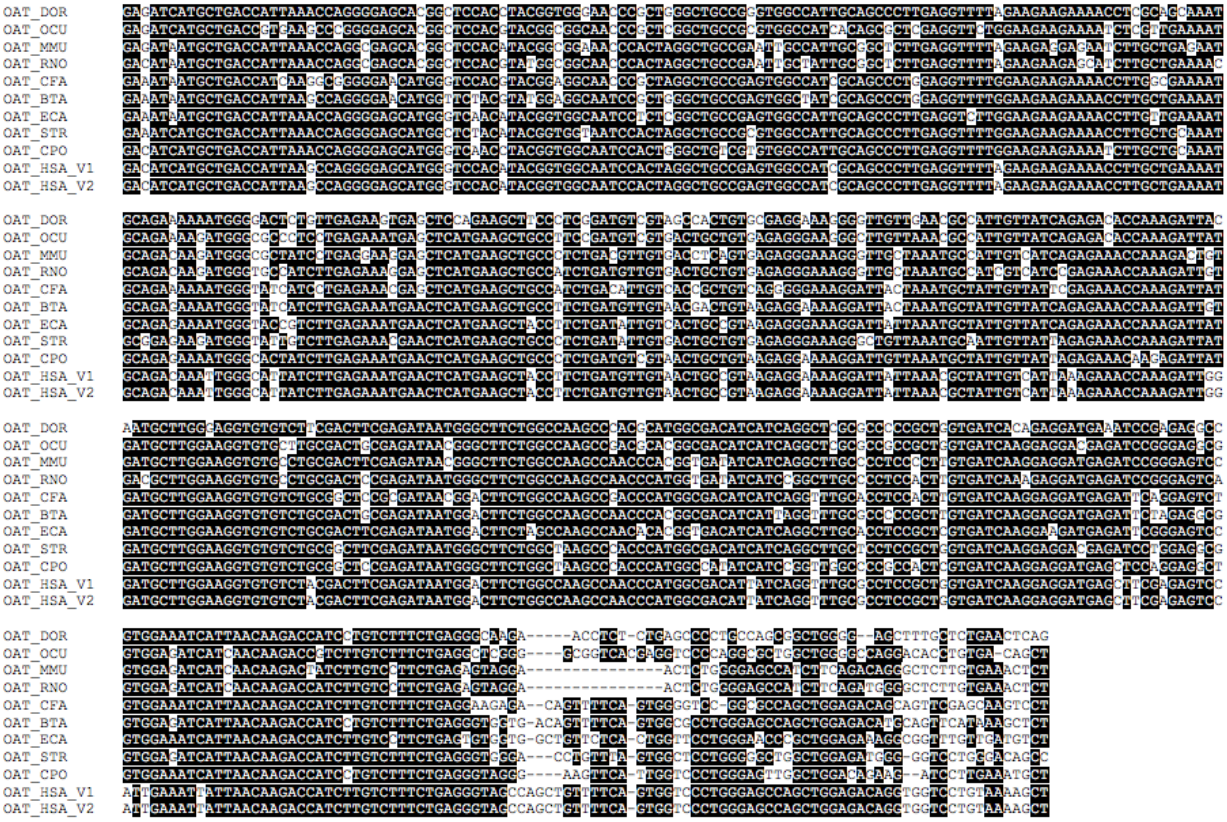
Appendix 1: Alignment of ALDH18A1 (P5CS) cDNA sequence from different species


ALDH18A1_DOR
ALDH18A1_STR
ALDH18A1_RNO
ALDH18A1_MMU_V1
ALDH18A1_MMU_V2
ALDH18A1_BTA
ALDH18A1_CFA
ALDH18A1_ECA
ALDH18A1_OCU
ALDH18A1_CPO
ALDH18A1_HSA_V1
ALDH18A1_HSA_V2

DOR	Dipodomys ordii	kangaroo rat
OCU	Oryctolagus cuniculus	rabbit
MMU	Mus musculus	mouse
RNO	Rattus norvegicus	rat
CFA	Canis familiaris	dog
BTA	Bos taurus	cattle
ECA	Equus caballus	horse
STR	Spermophilus tridecemlineatus	thirteen-lined ground squirrel
CPO	Cavia porcellus	guinea pig
HSA	Homo sapiens	man

Appendix 2: Alignment of OAT cDNA sequence from different species





DOR	Dipodomys ordii	kangaroo rat
OCU	Oryctolagus cuniculus	rabbit
MMU	Mus musculus	mouse
RNO	Rattus norvegicus	rat
CFA	Canis familiaris	dog
BTA	Bos taurus	cattle
ECA	Equus caballus	horse
STR	Spermophilus tridecemlineatus	thirteen-lined ground squirrel
CPO	Cavia porcellus	guinea pig
HSA	Homo sapiens	man

Appendix 3: Alignment of P5CS cDNA sequence from proline-auxotroph and –protroph CHO-K1

```
P5CS_CHO-K1_Pro-      1 atgttgagacacatgcatcgctctgggttccagcccttcaaccaacgccttttgccttgggtccagtccatagctgtgcccagatctcat
P5CS_CHO-K1_Pro+      1 atgttgagacacatgcatcgctctgggttccagcccttcaaccaacgccttttgccttgggtccagtccatagctgtgcccagatctcat

P5CS_CHO-K1_Pro-     91 ggagtccagccttcagctgtcagacatgttcgttcttggagcaacatccctttttatcacagtaccactcagtcgtgcccatggcaagccc
P5CS_CHO-K1_Pro+     91 ggagtccagccttcagctgtcagacatgttcgttcttggagcaacatccctttttatcacagtaccactcagtcgtgcccatggcaagccc

P5CS_CHO-K1_Pro-    181 tttgcccaccggagtgagctgaagcatgccaagagaattgtagttaaactcggcagtgccgtggtgaccagaggagatgagtgtggcctg
P5CS_CHO-K1_Pro+    181 tttgcccaccggagtgagctgaagcatgccaagagaattgtagttaaactcggcagtgccgtggtgaccagaggagatgagtgtggcctg

P5CS_CHO-K1_Pro-    271 gcactagggcgcctggcatctatcgtggaacaggtctcagtgctgcagaatcagggccgagagatgatgctggtcaccagcggagccgtt
P5CS_CHO-K1_Pro+    271 gcactagggcgcctggcatctatcgtggaacaggtctcagtgctgcagaatcagggccgagagatgatgctggtcaccagcggagccgtt

P5CS_CHO-K1_Pro-    361 gccttcggcaaacagcgcctgcgccatgagatccttctatctcagagtgtgcggcaggccctgcactcggggcagaaccagctgaaggag
P5CS_CHO-K1_Pro+    361 gccttcggcaaacagcgcctgcgccatgagatccttctatctcagagtgtgcggcaggccctgcactcggggcagaaccagctgaaggag

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P5CS_CHO-K1_Pro+    631 cttcggatgaatatcgtccccattgtcaacaccaacgatgctgttgtccccccagctgagcccaacagtgatcttcaggggtaaatgtt

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P5CS_CHO-K1_Pro+    721 attagtgttaaggataatgatagcctggctgcccgtctggccgtagaaatgaaaactgacctcttgattgtcctttccgatgtagaaggc

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P5CS_CHO-K1_Pro+    811 ctctttgacagccccccagggtcagatgatgcaaagctcattgatatcttctaccctggagatcaacagtcagtgacatttggaaccaaa

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P5CS_CHO-K1_Pro+   1081 cctacagtggagcagcagggagagatggctagatcaggaggaaggatgctggccacattggcgcctgaacagagagcagaaattatccat

P5CS_CHO-K1_Pro-   1171 cacctggctgacctgctgacggaccagcgggaagagatcctgctagccaacaaaaaagacttggaggaggcagagggaagacttgcgacc
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P5CS_CHO-K1_Pro+   1261 cctctaatgaagcgcctgagtctctccacatctaaactgaacagcctggccatcgggcttcggcagatcgcagcctcctcccaggacagt

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P5CS_CHO-K1_Pro+   1441 gagtctcgccctgactgtctacctcaggtggcagccttggctattgcaagtggcaatggtttgttgctcaaaggagggaaggaggccgca

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P5CS_CHO-K1_Pro+   1621 gaagtcgaggatctctgtcgcctcgacaaaataatagatctgatcattcctcgaggctcctcgcagctggtcagagacatccagaaagct

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P5CS_CHO-K1_Pro+   1711 gccaagggcatcccagtgatgggccacagcgaagggatctgccacatgtatgtggattccgaggccagtgtggacaaagtcaccagactc

P5CS_CHO-K1_Pro-   1801 gtcagggactccaagtgtgagtatccagctgcctgtaatgctttggagactttgctcatccaccgggatctgctgagaacacccttattt
P5CS_CHO-K1_Pro+   1801 gtcagggactccaagtgtgagtatccagctgcctgtaatgctttggagactttgctcatccaccgggatctgctgagaacacccttattt

P5CS_CHO-K1_Pro-   1891 gaccagatcatcgatatgctgcgagtggaacaggtaaaaattcatgcaggccccaagtttgcctcctacctgaccttcagcccctcagaa
P5CS_CHO-K1_Pro+   1891 gaccagatcatcgatatgctgcgagtggaacaggtaaaaattcatgcaggccccaagtttgcctcctacctgaccttcagcccctcagaa

P5CS_CHO-K1_Pro-   1981 gtgaagtcactccgaatagagtatggtgacctggaagtgtgcattgaagtggtggacagtgtccaggaagccattgatcacatccataaa
P5CS_CHO-K1_Pro+   1981 gtgaagtcactccgaatagagtatggtgacctggaagtgtgcattgaagtggtggacagtgtccaggaagccattgatcacatccataaa

P5CS_CHO-K1_Pro-   2071 tacggcagctcccacacagatgtcatcgtcacagagaacgagaaaacagcagagttcttcctccagcacgtggacagcgcctgtgtgttc
P5CS_CHO-K1_Pro+   2071 tacggcagctcccacacagatgtcatcgtcacagagaacgagaaaacagcagagttcttcctccagcacgtggacagcgcctgtgtgttc

P5CS_CHO-K1_Pro-   2161 tggaatgctagtactcgcttctctgatggctaccgctttggactgggagctgaagtaggaatcagtacatcaagaatccatgcccgggga
P5CS_CHO-K1_Pro+   2161 tggaatgctagtactcgcttctctgatggctaccgctttggactgggagctgaagtaggaatcagtacatcaagaatccatgcccgggga

P5CS_CHO-K1_Pro-   2251 ccagtaggactggaaggattgctaactactaagtggctgcttcgagggcaagaccatgtggtctcagacttctcagagcacggcagctta
P5CS_CHO-K1_Pro+   2251 ccagtaggactggaaggattgctaactactaagtggctgcttcgagggcaagaccatgtggtctcagacttctcagagcacggcagctta

P5CS_CHO-K1_Pro-   2341 aaatatcttcacgagaacctccctgttccccagagaaacaccaactga
P5CS_CHO-K1_Pro+   2341 aaatatcttcacgagaacctccctgttccccagagaaacaccaactga
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Appendix 4: Amino acid concentration in dialyzed FBS

Amino acid component	Short form	Concentraion in serum (nmol/mL)	others
Taurine	Tau	4.97	
Urea	Urea	608.56	
Aspartic acid	Asp	322.44	
Hydroxyproline	Hypro	4.65	Detected at λ= 440 nm
Threonine	Thr	322.34	
Serine	Ser	599.50	
Asparagine	Asn	189.00	
Glutamic acid	Glu	410.06	
Glutamine	Gln	194.64	
Proline	Pro	389.13	Detected at λ= 440 nm
Glycine	Gly	615.85	
Alanine	Ala	703.76	
Valine	Val	443.51	
Methionine	Met	153.88	
Isoleucine	Ileu	373.78	
Leucine	Leu	774.24	
Tyrosine	Try	240.71	
Phenylalanine	Phe	315.44	
β- aminobutric acid	β-Aba β-Aba	4.17 10.49	Two peaks in time frame of 0.3 min
γ- aminobutric acid	γ-Aba	6.57	
Histidine	His	144.08	
Tryptophane	Trp	129.10	
Hydroxylysine		665.42	
Ornithine	Orn	389.51	
Lysine	Lys	718.98	
Ammonia	NH3	311.92	
Arginine	Arg	346.30	

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